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# THE ACTION OF INSULIN

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# PREFACE

In this monograph, our aim has been to present the results of recent investigations dealing with the problem of the mechanism of insulin action in a manner which might interest the clinician as well as the research worker This discussion of the action of insulin should not be considered an all inclusive review of the subject, but rather a survey of the current state of our knowledge in this field, as we see it In so doing we have omitted much pertinent material, at the same time stressing those aspects of the action of insulin with which we have been directly concerned

To Dr William C Stadie, who has contributed so much to present knowledge of insulin action, we would like to express our thanks and warmly acknowledge our indebtedness We also wish to thank Dr F D W Lukens and Dr Richard B Singer for their helpful criticism

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# THE ACTION OF INSULIN

## CHAPTER I

# INTRODUCTION

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The experimental production in animals by surgical removal of the pancreas of a metabolic state closely resembling diabetes mellitus brought into being the problem of the action of insulin Many phases of this problem have been developed since its classical inception by von Mehring and Minkowski An early one was concerned with a study of the disturbances of total metabolism in the disease Experiments were largely confined to the intact animal *This period culminated with the development of* the two antithetical theories of under utilization and over production—now recognized to be over simplifications of complex phenomena The isolation of insulin as the active principle of the pancreas was the crowning achievement of this early work

But with the astounding developments in enzymology which revealed in detail the complex patterns of intermediary metabolism of foodstuffs the interest and aims of the metabolist working on diabetes shifted He now sought to localize the action of insulin to some particular reaction in the metabolic schema But the development of endocrinology has shown that insulin functions in a milieu of hormones and the elucidation of the chemical action of insulin continues to elude the ardent searches of many

investigators Thus the problem of the action of insulin has become one of the interaction of hormones with enzyme systems In time these interactions will yield their secrets to the researcher

A new phase is just beginning—that of studies of phenomena on the molecular level designed to reveal the molecular mechanism by which hormones and enzymes produce their catalytic effects Since little is known of these interactions in the case of enzymes alone it is not surprising that the added effects of hormones are shrouded in almost complete darkness No one can predict the limits to which continued research will lead But fascinating discoveries will continue to reward the diligent worker This monograph by Drs Haugaard and Marsh attempts to summarize our present knowledge of the problem of the action of insulin

## CHAPTER II

# ESSENTIALS OF INTERMEDIARY METABOLISM

SINCE the discovery of insulin, more than three decades ago, an extensive scientific literature on the subject of the action of insulin has accumulated This work has led to an understanding of metabolism in the diabetic organism and of the action of insulin on overall metabolic processes in the intact animal However, little progress has been achieved in the study of the problem of the mechanism whereby insulin exerts its manifold effects At present, the precise biochemical mode of action of insulin remains unknown The study of the mechanism of action of other hormones has similarly met with limited success

Since hormones are concerned with the regulation of metabolic reactions, progress in the study of hormonal action is dependent on the extent of our knowledge of intermediary metabolism For this reason we have chosen to begin our discussion with a consideration of some of the current concepts of cellular biochemistry

## ENDERGONIC AND EXERGONIC REACTIONS AND THE NATURE OF ENZYMATIC CATALYSIS

One of the chief characteristics of the living cell is its ability to accelerate chemical reactions which are ordinarily too slow to be easily demonstrable From these catalyzed reactions, energy is derived which is harnessed by the cell for the performance of work, growth and reproduction

The decrease in free energy ($-\Delta F$) associated with a chemical reaction is a measure of the maximum amount of energy which is available for the performance of work Reactions in which there is a total decrease in free energy are called exergonic Such reactions may occur spontaneously and the energy derived may be utilized for synthetic processes and work Endergonic reactions are associated with an increase in free energy They do not occur spontaneously but require energy to proceed

In the cell chemical reactions take place continuously in such a way that the energy derived from exergonic reactions is utilized to carry out endergonic processes Overall synthetic reactions such as the formation of glycogen fat or protein are endergonic The energy required to carry out these processes in the cell is obtained by linking the synthetic reactions to the oxidation of hydrogen to water a strongly exergonic reaction

*Although the value of* $\Delta F$ *for a given chemical reaction* indicates whether it is possible for such a reaction to occur spontaneously it does not yield information about the rate at which the reaction actually does occur under given experimental conditions To answer this question it is necessary to consider the activation energy of the reaction involved It is a familiar observation that an increase in temperature of 10° C will double or treble the rate of a chemical reaction The occurrence of a reaction depends upon the successful chance collision of two molecules or groups capable of reacting A rise in temperature increases the number of collisions and hence the reaction rate Yet actual calculation shows that a 10° rise in temperature will increase the average kinetic energy of most systems by 3% (1) hardly enough to explain a two-fold increase in reaction rate The explanation is that not all collisions between molecules result in a reaction Only collisions

between "active" molecules, i e, molecules having a total energy (chiefly vibrational) in excess of a certain amount do result in a reaction The amount of energy required to change one mole of average molecules into activated ones is called the activation energy, E It can be shown that a 10° rise in temperature doubles or triples the number of activated molecules

An excellent discussion of the significance of the activation energy in chemical reactions has been given by Haurowitz (2) It may be said that in a given reaction the overall change in free energy is not altered by a catalyst However, the catalyst decreases the activation energy by altering the pathway Thus the reaction (*1*) $A + B \rightleftharpoons AB$ may proceed by the pathway (*2*) $A + B + C \rightleftharpoons ABC \rightleftharpoons AB + C$ If the activation energy necessary for reaction (*2*) is lower than that necessary for reaction (*1*) C will act as a catalyst and the reaction rate will be accelerated From this point of view the action of an enzyme consists of changing the pathway of a reaction from one requiring a high activation energy to one requiring a low one

The work of Michaelis and Menten (3) led to the concept that enzymes combine with their substrates to form an enzyme-substrate complex which is subsequently broken down to the free enzyme and the products of the reaction The velocity of the overall reaction is determined by the rate of decomposition of the enzyme-substrate complex The intermediary compound corresponds to the formation of ABC in equation (*2*) above Direct proof for the existence of enzyme-substrate complexes has been obtained by Stern (4) and by Chance (5) for the enzyme catalase A mathematical expression of this concept of enzyme action is the Michaelis Menten equation in which the law of mass action is applied to the formation of the enzyme substrate complex The dissociation constant K

for the reaction enzyme + substrate⇌enzyme-substrate is equal to

$$\frac{\text{(concentration of free enzyme)}\ \text{(concentration of substrate)}}{\text{(concentration of enzyme substrate complex)}}$$

By appropriate mathematical treatment (6), this constant can be calculated for a given reaction from measurements of the reaction velocity as a function of substrate concentration The Michaelis constant is a measure of the affinity of the enzyme for the substrate

## THE STEPWISE UTILIZATION OF ENERGY

The energy provided by the reactions of intermediary metabolism is used by the cell for the performance of work, including growth and reproduction as well as mechanical work such as muscular contraction The overall process involves the oxidation of foodstuffs to $CO_2$ and $H_2O$ The energy so generated is not released all at once as it is when sugar is burned in air Instead, the original assimilated foodstuff molecules undergo a series of intermediate reactions In each step energy is absorbed or liberated by the synthesis or cleavage of the chemical bonds present in the intermediate compound Some of these steps are oxidative i e, hydrogen atoms (electrons and protons) are removed from the substrate The electrons are transferred by a series of respiratory enzymes and eventually cause the reduction of oxygen in the presence of hydrogen ions to form water

For example, the oxidation of lactic acid consists of a transfer of two electrons and two protons to a coenzyme, in this case, *diphosphopyridine nucleotide* (DPN) This reaction is catalyzed by a specific dehydrogenase The reduced coenzyme in turn oxidizes a second coenzyme, alloxazine adenine dinucleotide again with the help of a specific protein enzyme The protons (i e, $H^+$) transferred

to the coenzyme are exchangeable with protons in the environment so that what actually occurs is a transfer of electrons The final links in the chain of enzymes are cytochrome C and cytochrome oxidase These constitute an enzyme system containing iron in a porphyrin prosthetic group The iron is alternately oxidized and reduced The final step consists of the oxidation of cytochrome oxidase by molecular oxygen This series of reactions represents a pathway which is widely utilized by many cells for the oxidation of a variety of substrates However, the particular reactions and coenzymes involved vary with the type of cell and with the substrate oxidized Some substrates for example do not react with DPN but are oxidized directly by an enzyme containing alloxazine adenine dinucleotide as the requisite prosthetic group In some cells cytochromes other than cytochrome C are present

The important consequence of carrying out the oxidation of a substrate by a process such as the one described is that a maximum amount of energy may be obtained for useful work Each of the reactions involved take place in a nearly reversible manner so that very little energy is wasted

The essential principles involved in cellular oxidation are treated in detail in the book edited by Lardy (1) An excellent series of lectures by Dixon (7) is also pertinent to this discussion The problem of electron transfer in biological systems is discussed by Geissman (8)

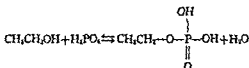
## THE MOBILIZATION OF ENERGY BY MEANS OF THE ENERGY RICH PHOSPHATE BOND

The researches of Harden and Young (9) on alcoholic fermentation in yeast and of Meyerhof (10) and others on glycolysis is mammalian muscle conclusively demonstrated

the importance of phosphorylated intermediates in cellular metabolism

The importance of the high energy phosphate bonds lies *in the realization that the chemical bond between the* organic part of the molecule and the phosphate radical may contain larger or smaller amounts of potential energy The compounds containing phosphate bonds of high potential energy serve as a store of energy which is readily *available when needed The continuous synthesis* and breakdown of high energy phosphate bonds constitute an important mechanism of intermediary metabolism

Let us consider the esterification of alcohol by phosphoric acid



In this reaction, ethyl alcohol is phosphorylated The reverse reaction would be called *dephosphorylation There* are other types of phosphate bonds which are not true esters The most important of these are

1 Carbonyl phosphate Example glucose-1 phosphate
2 Guanidine phosphate Example creatine phosphate
3 Carboxyl phosphate Example 1 3 diphospho-glyceric acid
4 Enol phosphate Example Phospho-pyruvic acid
5 Pyrophosphate Example The two terminal phosphate bonds in adenosine triphosphate

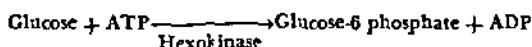
The four last mentioned of these bonds are high energy phosphate bonds The free energy contents of some of these bonds are listed below

Alcohol phosphate—400 calories per mole
Carbonyl phosphate—2,000 calories per mole

Pyrophosphate—10,500 calories per mole
Carboxyl or enol phosphate—14,000 calories per mole

The theoretical development of the concept of the energy rich phosphate bond is based to a great extent on the work of Lipmann (11) The subject has been reviewed by Kalckar (12), and Oesper (13) has discussed the difficult and interesting question of the relation between the molecular structure of the compounds involved and the energy content of the phosphate bonds

In the cell, few compounds are phosphorylated by a direct reaction with inorganic phosphate Instead most of them are *transphosphorylated* by reacting with a compound, usually ATP*, containing an energy rich phosphate bond The transformation of glucose to glucose-6 phosphate, the initial step of glucose metabolism, is such a reaction


Glucose + ATP ——Hexokinase——→ Glucose-6 phosphate + ADP

In this reaction, energy is supplied by the high energy phosphate bond of ATP and the resultant glucose 6 phosphate has a low energy phosphate bond The first step in the utilization of glucose—the hexokinase reaction—is a transphosphorylation reaction and it involves the expenditure of energy by the cell It seems paradoxical that the first step in the utilization of a foodstuff by the cell should require energy instead of yielding energy However the subsequent reactions of the glucose phosphate more than make up this deficit, since these reactions regenerate energy rich phosphate bonds in excess of the number initially introduced

* ATP Adenosine triphosphate
ADP Adenosine diphosphate

There are four main cellular reactions involving phosphate bonds (see Drabkin [14]). They are

1. Phosphorylation. Direct addition of inorganic phosphate.
   Example: Glyceraldehyde phosphate + DPN + phosphate ⇌ Glyceric acid diphosphate + $H_2$ DPN
2. Dephosphorylation. Splitting of the phosphate bond.
   Example: Glucose 6 phosphate + HOH $\underset{\text{phosphatase}}{\rightleftharpoons}$ Glucose + phosphate
3. Transphosphorylation. Transfer of phosphate from one compound to another.
   Example: Glucose + ATP $\xrightarrow[\text{hexokinase}]{}$ Glucose-6 phosphate + ADP
4. Phosphorolysis.
   Example: Glycogen + n phosphate $\underset{\text{phosphorylase}}{\rightleftharpoons}$ n Glucose 1 phosphate

Although the details have not been worked out as well for the metabolic reactions of protein and fat, there is ample evidence that phosphate plays an equally important role in their metabolism.

The energy accumulated in ATP is the driving force for synthetic reactions. As we have seen, this energy is obtained from degradative reactions which are mainly oxidative. It is important to note that the energy obtained in oxidative reactions is not only generated in the initial dehydrogenation of the substrate but that the subsequent reactions of the respiratory enzymes give rise to the production of energy rich phosphate bonds. This had been inferred from theoretical considerations (15) and was recently demonstrated experimentally by Friedkin and Lehninger (16).

There is yet another aspect of the conversion of metabolites into phosphorylated intermediates which should be considered. Drabkin (14) has pointed out that since the cell

contains approximately 70% water (40 molar) reactions yielding water which take place inside the cell do so against an overwhelming concentration gradient Con sider, for example the synthesis of glycogen The direct formation of glycogen by the polymerization of glucose would involve the production of water According to the law of mass action such a reaction would be difficult to accomplish since the high concentration of water in the cell favors the reverse reaction In the cell this reaction is accomplished by a preliminary phosphorylation of glucose to glucose 6 phosphate followed by transfer of the phos phate to the 1 position Glycogen is finally synthesized from glucose 1 phosphate a reaction involving the produc tion of inorganic phosphate rather than water Since the concentration of phosphate in the cell is low this reaction is much more easily accomplished

## THE INTERMEDIARY METABOLISM OF CAR BOHYDRATES, FAT AND PROTEIN AND THE CONCEPT OF THE METABOLIC POOL

As a result of extensive work by Schoenheimer (17) Stetten (18) and others with isotopically labeled metabo lites a dynamic concept of the nature of metabolic reactions has developed Before this work it was thought that fat for example was stored in stationary depots to be mobilized only when needed It is now clear that continual synthesis and breakdown of fat occurs even in the fat depots (19) It is apparent that a constant interchange of molecules be tween fat carbohydrate and protein takes place There is a metabolic pool of intermediate compounds which sup plies molecules for oxidation and for the synthesis of fat protein glycogen and all the other necessary components of the cell There is no sharp distinction between struc tural elements of the cell and substances used for the pro-

duction of energy by oxidation However, the rates at which different substances in the cell are broken down and rebuilt may vary considerably

The phosphorylated intermediates in the pathway of glycolysis constitute a metabolic pool. Regardless of the rate of glycolysis, the concentration of each intermediate at any given time is approximately the same While the rate of formation of end products may vary widely, the concen trations of the constituents of the metabolic pool are rela tively constant What does change with the rate of glycolysis is the speed with which the intermediates are formed and broken down This is called the turnover rate

The Krebs cycle is a fundamental sequence of reactions which begins with the reaction of oxaloacetate and a two carbon unit formed from acetate or pyruvate to form ci trate The chemical nature of the two-carbon fragment has recently been established as the acetyl derivative of coenzyme A (20) It is at this point that the interrelation between carbohydrate, fat and protein metabolism be comes apparent The acetyl-coenzyme A may be derived, ultimately, from fatty acids and amino acids as well as from carbohydrate Details of fat and protein metabolism will not be considered here, except to indicate that fatty acids are broken down into two carbon units which may form acetoacetic acid, or may be oxidized or resynthesized into fat It is interesting to note that $CO_2$ is not merely a waste product, but may be incorporated into organic com pounds by animal tissue even though excess $CO_2$ is even tually released

## ALTERNATIVE METABOLIC PATHWAYS

The cell provides many pathways for a given metabolite to follow For example, acetate may be oxidized via the Krebs cycle, may condense to form acetoacetate, or may

be used for the synthesis of fatty acids or cholesterol The pathway a given metabolite will take depends on the enzy matic pattern available and the concentrations of other metabolites present These factors in turn are dependent on the previous dietary and hormonal history of the ani mal and will vary with the type of cell involved In dis eases of metabolism such as diabetes in which it appears that certain metabolic pathways are blocked it may be possible for the cell to utilize other pathways to a greater extent than normal so that the energy requirements can be satisfied

## THE RELATION OF METABOLISM TO MORPHOLOGY

In the study of cellular metabolism one tends to over look the fact that the cell is composed of a nucleus cyto plasm and other structural elements Metabolic reactions do not occur in a haphazard jumble of enzymes and sub-strates Until recently however very little was known about the chemical composition of nuclei Golgi apparatus mitochondria and other structures so well known to the histologist This subject received an early impetus from the work of Linderstrøm Lang (21) Later simplified techniques of differential centrifugation worked out by Dounce (22) Claude (23) and others initiated an intensive study of the enzymatic properties of cellular constituents Essentially this method consists of homogenizing the tissue in solutions which tend to preserve the structure of the subcellular components and then subjecting the homog enates to centrifugation at different speeds Unbroken cells will be the first to sediment followed by the nuclei the mitochondria the microsomes and possibly other ele ments with the soluble cytoplasmic material remaining in the supernatant The reader is referred to the article

by Schneider for further details (24) The mitochondria appear to contain the enzymes of the cytochrome system and are able to carry out phosphorylative and synthetic reactions

It is possible that the mechanism of action of insulin and other hormones on cellular metabolism is intimately concerned with the problem of the arrangement of enzyme systems in structural units within the cell

## REGULATION OF THE RATE OF METABOLIC REACTIONS

One of the most important properties of the living cell is its ability to control the rate of metabolic reactions in such a way that the concentrations of metabolites are kept within closely defined limits Without this ability the substrates necessary for life would soon be used up While progress has been made in understanding the nature of the metabolic reactions carried out by the cell little is known about the mechanisms whereby the cell controls the rates of these reactions

Enzyme chemists have demonstrated that the rate of a given reaction catalyzed by an enzyme is dependent on the concentration of enzyme and substrate and is influenced by factors such as pH temperature and the concentration of certain inorganic ions and coenzymes. All of these variables may be involved in the regulation of metabolic reactions in the cell Since enzymatic reactions in living cells take place in heterogeneous systems additional mechanisms should be considered Among these are selective permeability of cell membranes to metabolites and ions the arrangement of enzymes in sub-cellular structures and rates of diffusion of metabolites to and from loci of enzymes Another factor involved in the regulation of cellular reactions well studied in bacteria is the ability of

the cell to change the effective concentrations of enzymes in response to metabolic needs

The currently accepted view concerning hormones is that these substances, which originate in cells especially developed for the purpose, influence tissue metabolism by enhancing or depressing metabolic reactions without being, themselves, catalytically active In addition, one hormone may oppose another in the process of regulating metabolic activity. This concept of interhormonal antagonism has proved fruitful in the study of hormone action, and as we shall see, is illustrated by the interplay of the anterior pituitary, adrenal cortical hormones and insulin in their effect on carbohydrate metabolism

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## CHAPTER III

# THE CHEMISTRY OF INSULIN

THE INSULIN molecule has been studied in such detail by chemical and physical methods that today we probably know as much about insulin as about any other protein Despite considerable information concerning the structure of insulin we cannot yet relate this knowledge to its physiological action

Early work on the chemistry of insulin has been discussed by Jensen (1) Since its publication new work has appeared much of which has been reviewed by Sanger (2)

## ISOLATION OF INSULIN

Before the successful isolation of insulin by Banting and Best in 1922 (3) several attempts had been made to obtain pancreatic extracts which were able to alleviate the symptoms of depancreatized animals or diabetic patients Early difficulties encountered in these attempts were due mainly to the destructive action of the proteolytic enzymes of the pancreas and to the fact that in many cases the extracts were given by mouth

The history of the investigations leading to the isolation of insulin has been related by MacLeod (4) Highly active extracts were obtained by the Montreal workers from the pancreatic glands of cattle by extraction with acid alcohol Collip (5) improved the methods of extraction and purification By the use of acid alcohol insulin was obtained in solution while most of the protein impurities were precipitated

Insulin was first crystallized by Abel (6) who used highly

purified amorphous preparations as a starting material Early difficulties encountered in attempts to crystallize insulin may have been due to the absence of salts of certain metals which aid in the crystallization Scott (7) observed that crystalline insulin contained zinc and that crystallization was easily obtained in the presence of salts of metals such as Zn Ni Co and Cd A discussion of modern methods of preparation of insulin has been published (8)

## GENERAL CHEMICAL PROPERTIES

Insulin yields only amino acids on hydrolysis It does not contain a prosthetic group The amino acid composition of insulin is not particularly different from that of most other proteins except perhaps that the content of cystine is high In native insulin no cysteine is found all of the sulphur being in the oxidized form

Insulin contains a large proportion of acidic and basic amino acids the molecule consequently contains many titratable acidic and basic groups It is quite soluble in water on either side of its isoelectric point (near pH 5 3) and can be dissolved in aqueous solutions of organic solvents

The figures for the molecular weight of insulin found by different authors vary considerably This may be explained by recent findings that crystalline insulin is a polymer of smaller units and that depolymerization occurs in solution The experimentally determined molecular weight which is a measure of the average molecular size will therefore vary with the experimental conditions

Gutfreund (9) using osmotic and centrifugal methods found that in concentrations of 0 4 0 9% and at a pH of 7 5 insulin solutions are homogeneous with respect to particle size and that the molecular weight of insulin in such solutions is 47 000-48 000 As we shall discuss later

this particle size corresponds to a polymer, the sub unit having been found to have a molecular weight of 12,000

## INACTIVATION OF INSULIN

Insulin is inactivated by relatively minor changes in the structure of the molecule. Treatment with 0.03 N sodium hydroxide for 3 hours at 34° C causes an irreversible inactivation with the simultaneous liberation of ammonia. In contrast to its lability under alkaline conditions insulin is very stable in acid. Insulin can be kept at 0° C in concentrated sulfuric acid for several hours without appreciable inactivation. When insulin is heated in acid solution a precipitate forms which is inactive, but activity may be restored by neutralization with alkali.

Insulin is inactivated by a number of reagents which react with specific groups present in the molecule. Reduction of the S S linkages causes inactivation. Table I shows the results obtained by Lens (10) in his studies on the inactivation of insulin by cysteine.

TABLE I*

*1% insulin, 0.25% cysteine pH 7.0 60% alcohol Temperature 30° C*

| *Time Minutes* | *% of cystine reduced* | *% activity left* |
|---|---|---|
| 0 | 0 | 100 |
| 5 | 2.7 | 60 |
| 15 | 7.3 | 20–35 |
| 60 | 10.2 | 13–14 |

* From Lens, J., and Neutelings J. *Biochem et Biophys Acta* 4 501 (1950)

It is interesting that the insulin had lost most of its activity when only a small fraction of the cystine in the molecule was reduced.

Stern and White (11) acetylated insulin *with ketene and* found that acetylation of the amino groups did not cause any decrease in activity After continued treatment with ketene *the phenolic groups became* substituted and the activity was lost Some of their results are given in Table II

TABLE II*

| *% amino groups acetylated* | *% phenolic groups acetylated* | *Physiological activity Units/ml* |
|---|---|---|
| 0 | 0 | 20–22 |
| 100 | 0 | 17–19 5 |
| 100 | 63 | 6 |
| 100 | 87 | 0 6 |

* From Stern, K G, and White, A *J Biol Chem*, *122* 371 (1938)

These experiments are in accord with recent experiments on the inactivation of insulin by enzymatic oxidation of the tyrosine residues with polyphenoloxidases (12)

Both S S linkages and the phenolic groups of the *tyrosine residues must be intact for the insulin molecule to* be active It is interesting to speculate on the question of whether these groups as such are involved in the mechanism of insulin action or whether the loss of activity following their destruction is the result of a change in the properties *of the molecule as a whole*

It has been known for some time that certain tissue preparations have the ability to inactivate insulin *in vitro* Schmidt (13) found that glycerol extracts of liver and kidney inactivate insulin after incubation at 37° for 18 hours Muscle extracts were found to be inactive Mirsky and Broh Kahn (14) also studied the inactivation of insulin by tissue extracts They postulated the existence of a specific insulin inactivating enzyme *which they called insulinase*

Liver and kidney extracts were found to have an especially high content of this enzyme The authors consider insulinase to have an important physiological function and found that the insulinase activity of tissue extracts varied with the diet The observation that liver extracts are capable of inactivating insulin is in accord with the experiments of Weisberg Friedman and Levine (15) who demonstrated that the intact liver is able to remove insulin from the blood stream It was estimated that the liver of the dog was capable of removing 1/25 to 1/50 of a unit of insulin per kilo body weight per hour

It is possible that insulinase plays an important role in the regulation of the insulin level in the organism However it has not yet been established whether the ability of tissue extracts to inactivate insulin reflects a corresponding ability of the intact tissues The degree of specificity of the enzyme also remains to be established

## THE PHYSICAL CHEMISTRY OF INSULIN AND THE STRUCTURE OF THE INSULIN MOLECULE

Recent work has led to further information concerning the structure of the insulin molecule The studies of Gutfreund (9) and of Oncley and Ellenbogen (16) have shown that the insulin molecule dissociates on dilution and in acid and alkali The minimum molecular weight observed was 12 000 ± 500 Such a molecular weight corresponds to that calculated from amino acid analysis Since insulin in more concentrated solutions at pH values of 4 0 to 7 5 has a molecular weight of 48 000 it appears that an equilibrium exists in solution between different forms of insulin At high concentrations the insulin exists predominantly as a tetramer On dilution depolymerization into sub units of molecular weight 12 000 takes place

Intermediate dimers and trimers probably also exist X ray measurements have shown that crystals of insulin have a molecular weight of 36 000 (17) and are *probably trimers* of the fundamental unit of 12 000

It is conceivable that at the low concentrations in which it is present in the body, insulin exists as a monomer If *this is true the active molecules* are smaller than was originally thought This small size, when compared with most protein molecules may explain in part the ease with which insulin enters the cells of the body

Under certain conditions insulin fibrils are formed when a solution of insulin is heated at pH 2 0 to 2 5 (18) The change is visualized by the formation of a thixotropic gel* which acquires static double refraction after an initial disturbance When the original gel is diluted, flow birefringence replaces the static birefringence Quantitative measurements of the flow birefringence show that the majority of the insulin molecules have been transformed into fibrils The visible heat precipitate of insulin obtained by heating solutions of insulin to 100° C in 0 1 N hydrochloric or sulfuric acid consisted of spherites which *were composed of insulin fibrils* When the precipitate was dissolved at pH 11 11 5 the solution had the typical double refraction of flow characteristic of insulin fibrils

Solid insulin fibers will remove insulin from solution This phenomenon *is the basis of a quantitative method* for the determination of insulin (19) A weighed insulin fiber is added to a solution containing insulin and the increase in weight after a period of equilibration is determined Only relatively large amounts of insulin can be measured by this procedure

The problem of the structure of insulin—i e, the way

* A thixotropic gel is one which liquefies after shaking

in which the constituent amino acids are linked together -is being studied in several laboratories by different ethods Butler and co-workers (20) split insulin by ymotrypsin and found that a number of polypeptides h molecular weights in the neighborhood of 800 were produced together with a larger core having a molecular weight range of 4 000 5 000 which was not attacked further Sanger (21) oxidized insulin with performic acid From studies of the resulting polypeptides he concluded that the insulin molecule with a molecular weight of 12 000 consists of four open polypeptide chains held together by S S bonds

Woolley (22) has reported the curious finding that the insulin molecule contains the bacterial growth factor streptogenin as part of a peptide chain

## SPECIES DIFFERENCES IN INSULIN

It was formerly thought that insulins isolated from lifferent species were chemically identical Sanger (23) nowever has recently found that the amino acid composition of insulin varies slightly from species to species He determined the amino acids present in ox pig and sheep insulins and found differences in the relative amounts of serine glycine threonine and alanine It is interesting that threonine is present in pig insulin and absent in the other insulins studied

## PREPARATION OF LABELED INSULIN

Reiner Keston and Green (24) and Ferrebee *et al* (25) have studied insulin labeled with $I^{131}$ Insulin has also been labeled by sulfation of aliphatic hydroxyl groups with sulfuric acid containing $S^{35}$ (26) according to the method of Reitz *et al* (27) Pettinga and Rice (28) reported that $S^{35}$ from labeled methionine was incorporated into

insulin produced by islet cells *in vitro* However, the specific activity of the isolated insulin was low

## RELATION OF THE STRUCTURE OF INSULIN TO ITS PHYSIOLOGICAL ACTION

It is possible that the sub unit of insulin with a molecular weight of 12,000 is the only form in which insulin is biologically active Indication that such is the case has been provided by experiments of Stadie and co-workers in which it was demonstrated that combination of insulin with tissue (see Chapter VIII) can be demonstrated at a pH of 2 At this pH insulin is known to be entirely dissociated into monomers It is also possible that the action of insulin in the body may involve a change from the globular to the fibrous state It must be admitted that the experimental conditions necessary to demonstrate such changes in the test tube are far removed from physiological conditions However, in the behavior of some of the muscle proteins we do have an example of a physiological mechanism involving the change of a protein from the globular to the fibrous state

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## CHAPTER IV

# THE PHYSIOLOGY OF INSULIN ACTION

## WHAT DOES INSULIN DO?

THE PROBLEM of the mechanism of action of insulin may be approached by defining the changes which take place after the administration of insulin to the intact animal It may be emphasized that the primary manifestation of insulin action *in vivo* is a lowering of the blood sugar level, and that diabetes mellitus is characterized by hyperglycemia and glycosuria

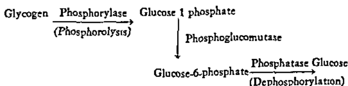
Insulin may produce hypoglycemia in one or both of two possible ways

(1) By decreasing the production of blood sugar by the liver,

(2) By increasing the utilization (ie, oxidation and storage) of glucose in the organs and tissues

## THE ROLE OF THE LIVER

The currently accepted reactions leading to the production of blood sugar from liver glycogen are as follows




If insulin causes hypoglycemia by diminishing the formation of glucose by the liver, it could conceivably do

so in two ways first by inhibiting the reactions leading from glycogen to glucose and second, by decreasing the formation of glucose from other precursors (inhibition of gluconeogenesis) What is the evidence that insulin has a direct effect on hepatic metabolism? This question has been investigated by Soskin and Levine (1) and by Bouckaert and DeDuve (2) The latter workers measured quantitatively the amount of glucose which disappears in the liver and in the peripheral tissues under the action of insulin This was done by determining the amount of glucose needed to maintain the blood sugar at a constant concentration following a standard maximum dose of insulin By comparing normal and hepatectomized animals with respect to the amount of glucose needed to maintain a constant level of blood sugar it was found that the liver accounted for a large fraction of the total glucose utilization These experiments are summarized in Table III

TABLE III*

| *No of Experiments* | *Operative deprivation* | *Insulin given* | *Glucose requirements to keep the blood sugar level constantly at its normal value of 0.87 Gm per liter* |
|---|---|---|---|
| | | | *As Gms glucose/Kg/80 mins* |
| 7 | None | + | 2.10±0.24 |
| 6 | Hepatectomy | + | 0.40±0.12 |
| 4 | Evisceration | + | 0.40±0.22 |

* From Bouckaert J P and De Duve, C *Physiol Rev*, 27 39 (1947)

It may be concluded from these results that insulin promotes the net uptake of glucose by the liver, since hepatectomy greatly diminished the amount of glucose necessary to maintain the blood sugar level after a large dose of in

sulin In a state of hypoglycemia, the liver must respond by restoring the blood sugar level The essential part played by the liver in this process makes it probable that an important aspect of the action of insulin is its effect on liver metabolism For reasons which we do not fully understand at present, however, attempts to demonstrate net effects of insulin *in vitro* on the carbohydrate metabolism of the liver have been unsuccessful

## INCREASED GLUCOSE UTILIZATION BY MUSCLE UNDER THE ACTION OF INSULIN

An increased utilization of blood glucose by the tissues will result in hypoglycemia assuming a constant rate of production by the liver There is no doubt about the ability of insulin to stimulate glucose consumption and glycogen synthesis in muscle It has been demonstrated in *the intact and eviscerated animal (2, 3) and in the isolated* rat diaphragm *in vitro* (4)

We may conclude that the primary physiologic effect of insulin, lowering of the blood sugar, is brought about by its action in increasing the utilization (oxidation and storage) of glucose in the organs and tissues of the body, and in decreasing the net production of glucose by the liver

## THE EFFECTS OF INSULIN ON FAT AND PROTEIN METABOLISM

The ketonuria and increased nitrogen excretion found in diabetes indicate a basic derangement of fat and protein metabolism With respect to fat metabolism we may summarize the effects of insulin (*1*) The excess production and excretion of ketone bodies in the diabetic is abolished by insulin (*2*) *In vitro* experiments with liver slices have disclosed that insulin inhibits the production of ketones

in diabetic cats (5) (3) Insulin accelerates the incorpora tion of $C^{14}$ labeled acetate into long chain fatty acids (6)

Insulin effects on protein metabolism include the in hibition of the excess protein breakdown in the diabetic and the suggestion (7) that insulin promotes protein synthesis *in vivo* This concept is based on the results of experiments in which insulin accelerated the disappear ance of amino acids from the blood stream in about the same proportions as these amino acids occur in muscle proteins (7)

The importance of adipose tissue in fat and carbohy drate metabolism long neglected in metabolic work is now being recognized An excellent review of this subject has been written by Wertheimer and Shapiro (8) The pre vention of the development of a fatty liver in the diabetic by administration of insulin has been described (9) It is not generally appreciated however that insulin may also increase the deposition of glycogen in the adipose tissue of normal rats (10) Fawcett (11) employing histochemical methods has found that the interscapular and perirenal fat is particularly susceptible to the action of insulin in promoting glycogen synthesis

## THE RELATIONSHIP OF BLOOD SUGAR LEVEL TO THE ACTION OF INSULIN

What is the physiologic significance of the hypoglycemic action of insulin? In keeping the blood sugar level rela tively constant insulin is only one of several hormones concerned The concentration of blood sugar is also affected by adrenal and pituitary secretions The elabora tion of insulin by the pancreas seems to be dependent on the blood sugar level Anderson and Long (12) showed that the secretion of insulin by the rat pancreas was increased during perfusion for one hour with hyperglycemic fluid

Dohan and Lukens (13) have shown that massive administration of glucose for a long time causes hydropic degeneration of the B cells, often accompanied by a diabetic state

The concept has been put forward by Soskin and Levine (1) that insulin enables the tissues to utilize glucose at lower glycemic levels than would be possible in its absence *In other words, diabetic hyperglycemia is an attempt,* in accordance with the law of mass action, to raise tissue glucose utilization and glycogen synthesis by raising the glucose concentration of the blood Soskin and Levine state that when one compares the rate of utilization of the normal animal at its usual normal blood sugar level with the rate of utilization of the diabetic animal at the hyperglycemic levels which it ordinarily maintains it is apparent that the diabetic animal habitually uses as much or more sugar than the normal animal "

If this hypothesis is accepted, one might expect that the effects of insulin on glycogen synthesis from glucose *in vitro* would tend to diminish as the concentration of glucose was raised However, experiments with the isolated rat diaphragm (14) indicate that the effect of insulin *increases* with increasing glucose concentration, even at a concentration of 1,000 mg per cent! In addition, recent experiments (15) have shown that when a constant hyperglycemic level of glucose $C^{14}$ is maintained, the rate of production *of respiratory* $C^{14}O_2$ *is lower than normal* in the alloxan diabetic rat In our opinion, these experiments constitute evidence against the concept that insulin is necessary only at low blood sugar levels

We may conclude this introduction *to the problem of* insulin action by emphasizing that the physiologic effects of insulin may be regarded in terms of the processes involved in the regulation of the concentration of glucose in the blood

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# CHAPTER V

# EXPERIMENTAL DIABETES

## PANCREATECTOMY

THE FUNDAMENTAL discovery of von Mehring and Minkowski (1) that the removal of the pancreas from dogs caused the appearance of diabetic symptoms indicated that diabetes mellitus in man was associated with the absence of a factor from the pancreas It was found that implantation of pancreatic tissue into depancreatized dogs alleviated the diabetic symptoms while removal of the transplants caused the reappearance of symptoms If the vascular supply of the pancreas was occluded the blood sugar rose and then fell when the ligature was removed It took thirty years from the time of the brilliant work of von Mehring and Minkowski before insulin finally was isolated by Banting and Best (2)

Dogs rapidly recover after removal of the pancreas They will survive for one to three weeks after the operation and show all the signs of diabetes hyperglycemia glycosuria ketonemia and ketonuria The RQ falls to about 0 7 even after feeding glucose The cholesterol content of the blood increases and there is a fall in the concentration of serum inorganic phosphate Excretion of nitrogen increases indicating an increased breakdown of protein There is usually a decrease in muscle glycogen while the glycogen content of the heart is increased

The pancreatectomized animal is deprived of pancreatic digestive enzymes as well as insulin In addition the depancreatized animal lacks the alpha cells of the Islets of

Langerhans which contain the newly discovered hyper glycemic factor (3) We do not know at present what the significance of this may be, but it emphasizes the fact that pancreatectomy is more than a state of insulin deficiency

## ALLOXAN DIABETES

Jacobs (4) in 1937 observed that if alloxan was injected into rabbits an initial hyperglycemia developed, followed by hypoglycemia The hypoglycemia could be prevented by the administration of glucose Dunn, Sheehan, and McLetchie (5) found that after the administration of alloxan to rabbits there developed an extensive necrosis of the Islets of Langerhans No changes in acinar tissue were noted All of the animals died within a few hours or days, the longest survival being five days None of the animals lived to develop permanent hyperglycemia and glycosuria Later work demonstrated that a permanent diabetic state could be produced in several species of animals by the injection of appropriate doses of alloxan (6, 7, 8)

The importance of this work lies in the discovery that alloxan, when given in small doses, acts perferentially on one type of cell The effect of thiouracil and related com pounds on the thyroid gland is another example of the specific action of a substance on a particular organ or cell (9) A further example is the action of tri ortho-cresyl phosphate (10) The administration of this substance to animals causes a degeneration of the myelin sheaths of the peripheral nerves, due to its specific affinity for the lipids of nervous tissue

These three compounds—alloxan, thiouracil and tri ortho cresyl phosphate, have the following properties in common (1) they exert their action on a specific tissue in which they cause histologically demonstrable changes, (2) they do not affect other tissues except at much higher con

centrations and (3) their activity is strongly associated with their particular chemical structure closely related compounds having less or no activity

Four phases in the development of the diabetic condition caused by alloxan can be distinguished (1) an initial hypoglycemic phase of short duration (2) a hyperglycemic phase lasting two to four hours followed by (3) a marked hypoglycemia of six to twelve hours duration and finally (4) a permanent hyperglycemia commencing 18 to 24 hours after the administration of alloxan

The initial changes in blood sugar concentration following alloxan appear to be of complex origin and agreement on the mechanisms by which they are brought about has not been reached The subject has been studied in detail by Wrenshall Collins-Williams and Best (11) and by Brunfeldt and Iversen (12) There is evidence that the temporary hyperglycemia is related to the production of glucose by the liver A release of insulin from the pancreas may be responsible for the hypoglycemic phase which precedes the final hyperglycemia

Several substances have been found to protect against the action of alloxan if injected immediately prior to or simultaneously with the alloxan Reduced glutathione cysteine and other sulfhydryl containing substances have been found especially effective These compounds probably inactivate alloxan in the blood stream and prevent its effect on the pancreas Leech and Bailey (13) found that alloxan when injected into rabbits was rapidly destroyed and that there was a concurrent decrease in the blood glutathione content Lazarow (14) demonstrated that both glutathione and cysteine prevented the development of alloxan diabetes. If the sulfhydryl compounds were administered three minutes after the injection of alloxan no protection was found British Anti Lewisite (BAL) which

combines with alloxan has also been found to give complete protection against its diabetogenic action (15) The composition of the diet of the experimental animal in the period preceding the injection of alloxan is also important Fasted rats are more sensitive to the action of alloxan than fed rats (16) There are also species differences in the susceptibility of animals to alloxan (17)

## SIDE EFFECTS OF ALLOXAN

Although the primary action of alloxan is on the beta cells of the Islets of Langerhans other effects are also found especially with high doses

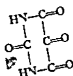
The question of whether alloxan itself has an effect on metabolism has not been adequately answered Possibly some of the changes in metabolism found in alloxanized animals may be caused by a direct action of alloxan In large doses alloxan causes an acute necrosis of the kidney tubules The kidney lesions may be reversible Some changes have been reported in other organs notably the liver and adrenals Brunfeldt and Iversen (12) reported the production of massive hepatic injury after direct perfusion of the liver with alloxan There was only a slight effect of the alloxan on liver glycogenolysis It is doubtful whether the concentration of alloxan after administration to the intact animal ever reaches levels as high as those used in the perfusion experiments Canzanelli Guild and Rapport (18) found no effect of alloxan on the rate of glycogenolysis in liver slices An effect of alloxan on the rate of the hexokinase reaction has been described (19) Since alloxan is rapidly broken down in the intact animal such effects are probably not concerned with the development of diabetes

The reactions involved in the breakdown of alloxan in the animal body have not been clearly established It is pos

sible that some of the degradation products of alloxan have specific metabolic effects that may persist for a long time Alloxan may also have effects on endocrine organs other than the pancreas that are not recognized at present and which might affect the metabolism of alloxanized animals

## EFFECTS OF SUBSTANCES STRUCTURALLY RELATED TO ALLOXAN

Alloxan has the following structure




It is a pyrimidine and therefore related chemically to the naturally occurring purines and pyrimidines

Bruckman and Wertheimer (20) have studied the possible diabetogenic action of substances structurally related to alloxan They came to the following general conclusions (*1*) An intact pyrimidine nucleus is essential for diabetogenicity, (2) activity is abolished by substitution anywhere in the alloxan molecule except in *one* of the imino groups (*3*) if the substituted side chain contains more than three carbon atoms the activity is abolished, (*4*) the absence of an ionizable hydrogen ion may explain the lack of activity of disubstituted alloxans such as dimethyl alloxan Since the disubstituted alloxans cause severe kidney damage it is clear that the islet cell and kidney lesions are not produced by the same chemical groupings Substances such as alloxantin dialuric acid and other derivatives which can be split *in vivo* to produce alloxan are also capable of producing diabetes

Little is known about the mode of action of alloxan in producing its effect on the beta cells (17) Several hypothe

ses have been advanced (1) Alloxan acts by selectively accumulating in toxic doses in the beta cells (2) Alloxan inhibits an enzymatic reaction in the beta cell by competing with a structurally related natural substrate (3) Alloxan inhibits a specific enzyme by reacting with its sulfhydryl groups

## RELATION OF THE ACTION OF ALLOXAN TO THE ETIOLOGY OF DIABETES

The thought has occurred to many that a relation may exist between the action of alloxan and the etiology of diabetes So far there has been no unequivocal demonstration that alloxan itself occurs naturally in the animal body

Seligson and Seligson (21) have shown that alloxan is rapidly broken down to alloxanic acid in human plasma or in the body When alloxanic acid is treated with alkali it forms oxomalonic acid, the carbon moiety of alloxan They (22) have been able to isolate the oxomalonate as the 2,4 dinitrophenylhydrazone from human urine after hydrolysis This indicates that alloxanic acid or a closely related compound is normally excreted in man The possible relation of this finding to the pathogenesis of human diabetes is now being investigated

Lazarow (23) has discussed the various factors which may influence the onset and progression of diabetes mellitus He emphasizes particularly the role of glutathione and discusses the possibility that the faulty metabolism of this substance may result in diabetes

## PITUITARY DIABETES

Temporary hyperglycemia, glycosuria, ketonuria, and other manifestations of diabetes can be produced by starvation, overfeeding, and the administration of various hormones The production of experimental glycosuria in the

rat has been discussed at length in a critical review by Ingle (24)

The relation of the pituitary gland to diabetes was first realized when Houssay and collaborators (25) found that extirpation of the pituitary of depancreatized animals resulted in an amelioration of the diabetic symptoms Implantation of the gland or injection of anterior lobe extracts into hypohysectomized depancreatized animals greatly increased the severity of diabetes In normal animals injection of anterior pituitary extract caused a temporary hyperglycemia and an increased resistance to the action of insulin The effects were transitory and the animals returned to normal after cessation of treatment

Young (26) was the first to show that when massive doses of anterior pituitary extract were given to dogs a permanent diabetes could be produced The pituitary-diabetic dogs differ from pancreatectomized dogs in that they live longer without insulin therapy and their fasting blood sugar is lower These animals have adequate amounts of pancreatic enzymes and are better able to utilize non carbohydrate food After the isolation of the growth hormone in a purified state it became possible to test its diabetogenic activity Cotes Reid and Young (27) reported the production of diabetes in cats by growth hormone administration and Houssay and Anderson (28) demonstrated diabetogenic activity of the purified growth hormone in dogs cats and batrachians Campbell and coworkers (29) described experiments in which permanent diabetes was obtained in dogs by growth hormone administration In one of their experiments a dog was given 3 mgs of growth hormone per kilo per day for 27 days For 6 weeks after the termination of this treatment the average daily excretion of glucose was 174 grams

The recent important observations on the effect of

growth hormone on carbohydrate metabolism and its relation to the action of insulin will be discussed in a later section concerned with the relation of insulin to pituitary and adrenal cortical hormones

The mechanism of the diabetogenic action of growth hormone is not known One possibility is that the presence of excess growth hormone in the body brings forth an increased demand for insulin causing excessive insulin production by the beta-cells and resulting in exhaustion atrophy Anderson and Long (30) have investigated the problem of whether there is any direct action of the growth hormone on the pancreas They studied the isolated pancreas of the rat in a small perfusion apparatus and found that the addition of growth hormone to the blood used for the perfusion caused a decrease rather than an increase in the secretion of insulin When hyperglycemic blood was given insulin secretion increased On the other hand the experiments of Millman and Russell (31) in which the injection of growth hormone produced hypoglycemia in normal rats but hyperglycemia is alloxanized or depancreatized rats suggest that the administration of growth hormone may result in the secretion of extra insulin

## ADRENAL CORTICAL HORMONES

A temporary glycosuria has been produced by the administration of ACTH to rats fed a high carbohydrate diet (32) Glycosuria and hyperglycemia were also obtained in similar experiments after the administration of 17 hydroxycorticosterone and corticosterone The glycosuria was associated with an increased excretion of nitrogen indicating increased gluconeogenesis No permanent diabetic condition developed in any of the animals used for these experiments Kobernick and More (33) however

recently described the hydropic degeneration of the beta cells of the pancreas in rabbits which had received a daily injection of 20 mgs of cortisone for several days The blood sugar rose steadily and large increases in the cho lesterol and fatty acid content of the blood were observed Thorn *et al* (34) have found that the 11 oxygenated adrenal cortical steroids and ACTH evoked hyper glycemia and glycosuria in man

## EFFECT OF CONTINUOUS HYPERGLYCEMIA

Dohan and Lukens (35) found that degeneration of the beta-cells of the pancreas and permanent diabetes may be obtained in cats by the continuous administration of car bohydrate Cats were given large doses of glucose in traperitoneally for long periods of time In several in stances hyperglycemia, glycosuria and ketonuria continued after the cessation of treatment The lesions of the beta cells were related to the degree and especially to the duration of the hyperglycemia Apparently hyperglycemia, by causing an excessive secretion of insulin, was responsi ble for the beta cell destruction It is possible that a similar mechanism is involved in the diabetogenic action of pituitary and adrenal cortical hormones

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## CHAPTER VI

# DIABETIC METABOLISM

Metabolism in diabetes mellitus has been a subject of clinical and laboratory investigation for more than half a century There was little progress made in the understanding of this disorder until the experimental production of a diabetic state by pancreatectomy was achieved by von Mehring and Minkowski and the nutritional experiments of Lusk and others were begun More recently the use of alloxan as a diabetogenic agent has facilitated the investigation of diabetic metabolism Despite the amount of information which has accumulated there is still no agreement concerning the primary defect in metabolism

For many years the discussion centered around two opposing theories under utilization and overproduction of glucose According to the under utilization theory the primary reason for the accumulation of glucose in the blood of the diabetic is a decreased ability of the organism to utilize glucose The theory of overproduction postulates that in diabetes the production of glucose from protein and fat is increased to such an extent that the organism is unable to metabolize all that is produced

Recently many experiments have been reported in which the question of the metabolic defect in diabetes has been re examined largely with the aid of isotopes Although these investigations have not solved the problem several important advances have been made Convincing evidence has been obtained which shows that oxidation of glucose

is depressed in the diabetic state and that diabetes is associated with an impairment of fat synthesis

In most of these experiments, alloxan-diabetic rats were used In general, alloxanized animals exhibit metabolic alterations similar to those in depancreatized animals and in human diabetics, although there are important differences in these three metabolic states Alloxanized rats with hyperglycemia, polyuria and glycosuria have diminished muscle glycogen (1) and an increased content of glycogen in the heart (2) The fasting level of liver glycogen appears to be above normal (3) *In vitro* studies on the metabolism of the diaphragm from the alloxan diabetic rat have shown that this tissue has a subnormal rate of glucose utilization (4) and glycogen synthesis (5) In Table IV we see the results of a series of experiments in which the respiration and glycogen synthesis of diaphragms from normal and alloxan diabetic rats were studied Alloxanization causes a decrease in glucose uptake

TABLE IV

OXYGEN UPTAKE, RG, AND GLYCOGEN SYNTHESIS BY THE ISOLATED DIAPHRAGM FROM NORMAL AND ALLOXANIZED RATS*

Two hour equilibration in phosphate-saline medium containing 0 2% glucose All animals had a blood sugar at the time of sacrifice greater than 509 mg %

| | *No of experiments* | *Normal* | *Alloxanized* | *Significance of difference (P)* |
|---|---|---|---|---|
| *Oxygen Uptake* Micromoles/Gm/2 hrs | 10 | 48±5 2 | 45±4 0 | — |
| *RQ* | 8 | 0 89±0 017 | 0 78±0 024 | <0 01 |
| *Glycogen Synthesis* Micromoles glucose equivalents/Gm/2 hrs | 8 | 9 7 ±0 98 | 4 7 ±0 74 | 0 001 |

* *Haugaard, N and Stadie, W C Unpublished data*

and glycogen synthesis and a lowering of the RQ in the isolated rat diaphragm

Stetten *et al* (6) have recently succeeded in demonstrating that the oxidation of $C^{14}$–glucose to $C^{14}O_2$ is diminished in alloxan diabetic rats Similar results were obtained by Feller and co workers (7) in studies using depancreatized dogs These observations are in accord with those of Wick *et al* (8) who demonstrated that the oxidation of radioactive glucose to $CO_2$ in the eviscerated rabbit was increased by insulin

In the experiments of Stetten and co-workers (6) radioactive glucose was injected into rats at a steady rate for several hours and the radioactivity of the expired $CO_2$ and of the glucose excreted in the urine was measured At the end of the experiment the radioactivity of the liver glycogen was also determined The authors came to the conclusion that alloxanization caused a significant decrease in the amount of glucose oxidized and an increase in the production of glucose from non-carbohydrate precursors These experiments clearly indicate that both an increased formation and a decreased utilization of glucose are factors contributing to the metabolic state in diabetes

Hastings Villée and co workers have studied the effect of insulin on the carbohydrate metabolism of isolated tissues using isotopically labeled metabolites With isolated rat diaphragm (9) insulin increased the utilization of glucose and the formation of glycogen and $CO_2$ from labeled glucose These reactions proceeded at a rate lower than normal in diaphragms from alloxan diabetic rats The rates of oxidation of acetate and pyruvate (10) to $CO_2$ were diminished in diaphragms from alloxan-diabetic rats The rate of pyruvate oxidation was restored to normal by insulin while the oxidation of acetate was unaffected

The oxidation of labeled pyruvate to $CO_2$ was found to be decreased in heart slices from diabetic animals, but addition of insulin did not restore pyruvate oxidation to normal in this tissue (11) Carbohydrate metabolism was also studied in rat liver slices using similar methods (12)

The authors conclude that their experimental findings are *in accord with the theory that* insulin produces a stimulation of the hexokinase system and that it has an additional effect on one or more of the reactions involved in the condensation of pyruvate with oxaloacetate to enter the citric acid cycle (13)

## FAT METABOLISM

It has been known for some time that the fat depots of alloxanized or depancreatized animals, as well as those of human diabetics rapidly dwindle Faulty metabolism of fat is also evident in the accumulation of ketone bodies in the diabetic The ketone bodies are not toxic by products of diabetic metabolism but are actively metabolized by normal or diabetic tissue This subject has been studied by Blixenkrone-Møller (14) in perfused livers and by Stadie, Zapp and Lukens (15) in rat and cat liver slices In diabetes, there was an increased production of ketones by the liver but the oxidation of ketone bodies by the extra hepatic tissues was unimpaired after pancreatectomy Stadie *et al* (15) showed that liver slices from depancreatized hypophysectomized cats produced ketone bodies at a much lower rate than slices from depancreatized cats Prior treatment of a depancreatized animal with insulin reduced the subsequent ability of the liver slice to produce ketones An effect of insulin in depressing the production of ketone bodies by rat liver slices in the presence of fructose and fumarate was demonstrated

Pauls and Drury (16) postulated that insulin promoted the utilization of glucose for fatty acid synthesis Depancreatized rats were maintained on a high carbohydrate diet with and without insulin The intake and excretion of glucose was measured over a period of time and the amount of carbohydrate retained was calculated The animals were then sacrificed and the liver and carcass glycogen determined It was found that insulin increased the storage of sugar as glycogen in the liver and muscles of the diabetic rat However, only about one fourth of the extra glucose that was retained was accounted for as glycogen Measurements of the oxygen consumption and nitrogen excretion excluded the possibility that a major portion of the extra glucose retained after insulin could have been oxidized or stored as protein The authors concluded therefore, that the extra sugar had been converted to fat

The recent availability of isotopic compounds has stimulated further studies of fat metabolism in diabetes Stetten and Boxer (17) studied the formation of glycogen and fatty acids by livers of normal and alloxan-diabetic rats Heavy water was administered and its rate of incorporation into the glycogen and fatty acids of the liver was determined Since the formation of glycogen and fat involves the addition of water from the surrounding medium, calculations of rates of synthesis can be made from such measurements In the alloxanized rat, fatty acid synthesis was decreased to 5% of the rate in normal rats and the glycogen was formed predominantly from smaller units such as lactate rather than from glucose directly Stetten and Boxer concluded that a major metabolic defect in the diabetic organism is its inability to synthesize fat from carbohydrate It was suggested that insulin may not

play a specific role in lipogenesis and that its effect on fat synthesis may be secondary to its effect on the utilization of glucose

Block and Kramer (18) and Brady and Gurin (19) measured the incorporation of labeled acetate into fatty acids in surviving rat liver slices They found that insulin produced a significant increase in the synthesis of fat from acetate by normal liver slices Brady and Gurin (20) in later experiments found that liver slices from alloxan diabetic rats had almost completely lost their ability to synthesize long-chain fatty acids from acetate Oddly enough, the addition of insulin did not increase fatty acid synthesis in the liver slices from these diabetic rats Brady, Gurin and Lukens (21) studied the ability of cat liver slices to incorporate labeled acetate into fatty acids In agreement with their observations with alloxanized rats liver slices from depancreatized cats were found to synthesize fat from acetate at a very low rate Addition of insulin to the medium had no effect in restoring the rate of fat synthesis from acetate It was also concluded that fat synthesis and the effect of insulin were limited by the presence of a pituitary factor, since hypophysectomy of the depancreatized animals restored the ability of the liver to synthesize fat from acetate and respond to insulin

Chernick *et al* (22) demonstrated a decreased synthesis of fat from $C^{14}$ labeled glucose as well as a decreased oxidation of glucose to $CO_2$ in liver slices from alloxanized rats Pretreatment of the diabetic rats with insulin for one to five days completely repaired this inability to utilize carbohydrate for lipogenesis When fructose was used as a substrate instead of glucose there was also a decreased rate of fat synthesis but the oxidation of fructose was not affected The authors concluded that there are two metabolic blocks in the diabetic liver—one involving the

conversion of glucose to fructose 6 phosphate and the other in the formation of fatty acids from "two carbon like" intermediates It is difficult to evaluate this conclusion at the present time

More recent experiments of Chaikoff and co workers (23) on the metabolism of liver slices from alloxan-diabetic rats suggest that glucose metabolism is blocked at an early stage When diabetic rats were fed a diet high in fructose, the subsequent ability of liver slices to synthesize fat from acetate (but *not* from glucose) was restored to normal Pre feeding a high glucose diet was not effective The authors conclude that the decreased ability of the diabetic liver slice to synthesize fat from acetate is a secondary effect related to impairment of glucose metabolism Feeding of fructose, which is readily metabolized by the diabetic liver, repairs the damage to fat metabolism while glucose, unable to enter the metabolic pool, is without effect

*We may summarize the results of these studies by stating that the most important metabolic defects in diabetes are in the synthesis of fat and in the utilization of carbohydrate*

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CHAPTER VII

# THE RELATIONSHIP OF ANTERIOR PITUITARY AND ADRENAL CORTICAL FACTORS TO THE ACTION OF INSULIN

THE ALTERATIONS in metabolism revealed by the study of experimental diabetes have disclosed the participation of adrenal and pituitary factors in the regulation of carbohydrate metabolism. Houssay (1) discovered the amelioration of diabetes subsequent to hypophysectomy. Long and Lukens (2) demonstrated that adrenalectomy likewise lessened the severity of the diabetic state. Conversely, Young (3) succeeded in producing permanent diabetes by the injection of anterior pituitary extract while Ingle (4) has rendered animals glycosuric with adrenal steroids. Conn (5) has produced temporary diabetes in man by the use of pituitary adrenocorticotrophic hormone (ACTH). These experiments indicate that adrenal and pituitary hormones oppose the action of insulin.

The metabolic functions of these endocrine glands may be studied *in vivo* by following the changes attendant upon removal of the gland and administration of glandular extracts, or they may be investigated by *in vitro* techniques, measuring effects on the metabolism of isolated tissue.

## *IN VIVO* STUDIES—THE METABOLIC EFFECTS OF HYPOPHYSECTOMY AND ANTERIOR PITUITARY EXTRACT ADMINISTRATION

The outstanding characteristic of the hypophysecto mized animal is a diminution in function of all other endocrine glands There is loss of gonadal function and the secondary sex characteristics, hypothyroidism, and susceptibility to stress characteristic of adrenal cortical failure It is therefore difficult to decide just how many of the observed metabolic defects are due to lack of pitui tary factors *per se*, and how many to lack of adrenal thy roid or gonadal secretion

From the standpoint of carbohydrate metabolism the hypophysectomized animal shows a marked inability to maintain its store of muscle and liver glycogen after fast ing Death may therefore result from hypoglycemia After glucose administration, less glycogen is deposited in the liver than in normal animals (6) The hypophysectomized animal is markedly sensitive to insulin This hyper sensitivity may mean that less insulin is required to ac celerate glucose uptake by the tissues or diminish hepatic glucose output than in the normal animal Since eviscer ated hypophysectomized rats are still hypersensitive to insulin (7) part of the hypersensitivity must be due to the absence of some factor in the peripheral tissues normally opposed to the action of insulin However, Crandall and Cherry (8) have demonstrated that insulin was more effec tive in reducing hepatic glucose production in hypophy sectomized dogs than in normals This indicates that both the liver and the peripheral tissues contribute to the insulin hypersensitivity of the hypophysectomized animal

The inability of the hypophysectomized animal to main

tain its carbohydrate depots after fasting suggests an exces sive utilization of carbohydrate This is also indicated by the rise of RQ following hypophysectomy Direct proof of increased glucose utilization was obtained by Russell (9) who showed that more glucose was necessary to main tain the blood sugar of eviscerated hypophysectomized animals than eviscerated controls The adrenalectomized animal likewise displays an inability to store liver glycogen and develops fasting hypoglycemia These effects of hy pophysectomy may therefore be related to secondary adrenal cortical insufficiency

The administration of crude anterior pituitary extracts restores the hypophysectomized animal to a comparatively normal state Russell (10) has shown that anterior pitui tary extract will preserve the level of muscle glycogen after fasting the so called glycostatic effect Crude extracts will also *promote the retention of nitrogen* In large doses anterior pituitary extract (APE) produces a permanent diabetic state (3) If diabetes is already present APE in creases glycosuria

What substances in the anterior pituitary are responsi ble for these results? With the recent isolation and purifi cation of the growth (11) and adrenocorticotrophic hor mones (12) it has become possible to provide a partial answer to this question

## THE METABOLIC EFFECTS OF PURIFIED GROWTH AND ADRENOCORTICOTROPHIC HORMONES

Many of the diabetogenic properties of crude pituitary extracts appear to be attributable to the growth hormone (13) The extracts used by Young (3) to render his animals permanently diabetic were high in growth hormone con tent The growth hormone (GH) has been crystallized (11,

14) and the crystalline material was found to increase the glycosuria of depancreatized rats and to enhance ketosis (13, 16) Recently (15) a permanent diabetic state has been produced by the administration of crystalline growth hormone to cats Either ACTH or GH will increase the urinary excretion of ketone bodies in normal rats fasted for three days (16) However, GH promotes nitrogen reten

TABLE V*

COMPARISON OF THE METABOLIC EFFECTS OF PURIFIED GROWTH AND ADRENOTROPHIC HORMONES

| | |
|---|---|
| 1 Promotes growth | 1 Inhibits growth |
| 2 Lowers R Q | 2 Effect on R Q doubtful |
| 3 Ketogenic | 3 Ketogenic† |
| 4 Nitrogen retained | 4 Nitrogen unchanged or lost |
| 5 Lowers blood amino acids | 5 Amino acids unchanged |
| 6 BUN and NPN unchanged | 6 Increases BUN and NPN |
| 7 *Liver arginase unchanged* | 7 *Increases liver arginase* |
| 8 Maintains muscle glycogen (short experiments) | 8 Maintains muscle glycogen (long experiments) |
| 9 Lowers pancreatic insulin | 9 Increases pancreatic insul n |
| 10 Slightly increases glycosuria of partially depancreatized rats | 10 Markedly increases glycosuria of partially depancreatized rats |

* Lukens F D W *Am J Med Sci*, *212* 229 (1946)

† In the original table, it was inferred that ACTH had no ketogen c effect but since then, Bennett *et al* (16) have found such an effect

tion while ACTH increases nitrogen excretion (17) Al though the growth hormone has been crystallized recent observations indicate that it may contain more than one factor For example Tepperman and Tepperman (18) found that the ability of growth hormone preparations to stimulate ketogenesis in rat liver slices did *not parallel* their ability to stimulate growth

The maintenance of muscle glycogen after fasting (the glycostatic effect) by *crude* APE occurs in the absence of

the adrenals and this property is possessed by growth hormone preparations If ACTH is given to the animal during the fasting period (usually 24 hours) the level of muscle glycogen drops as usual But if the animal is pretreated with ACTH to stimulate the atrophic adrenals and then fasted the muscle glycogen content is maintained This indicates that GH and ACTH exert a glycostatic effect on muscle glycogen by two different mechanisms Perhaps ACTH acts to promote carbohydrate storage by liberating the appropriate adrenal steroids while the growth hormone may preserve muscle glycogen after fasting by slowing down the oxidation of carbohydrate in the muscle A comparison of the metabolic effects of purified growth and adrenocorticotrophic hormones has been made by Lukens (19) and is reproduced in Table V

## THE INSULIN ANTAGONISTIC EFFECTS OF *CRUDE APE, AND PURIFIED* GH AND ACTH

From the *in vivo* studies already mentioned it is apparent that anterior pituitary hormones oppose the action of insulin It follows that the hypophysectomized animal should be hypersensitive to insulin For many years it was wondered whether changes in the islet cells following administration of pituitary extracts might result from a direct stimulatory or destructive action of the pituitary (20) While this has not been disproven it is now felt by many workers that hydropic degeneration may represent an exhaustion of the beta cells caused by an excessive insulin secretion This shifts the emphasis concerning the anti insulin effect of the pituitary to its action on the tissues especially liver and muscle

Which hormone is responsible for the anti insulin effect

of crude APE? So far, highly purified growth, adreno corticotrophic, lactogenic, thyrotrophic, follicle stimulating and luteinizing hormones have been isolated from anterior pituitary extracts Only the growth (21) and adrenotrophic hormones appear to possess insulin antagonistic properties It is clear from what has already been said that there are important differences in the properties of these two hormones As far as we know, ACTH must produce its effect by the *liberation of steroid hormones* from the adrenal cortex, while growth hormone has no adrenotrophic action Yet, as we will see, the effects of the *growth hormone are diminished in adrenalectomized* animals It appears likely that there exists a synergistic relationship between pituitary and adrenal hormones with respect to their insulin antagonistic properties

## THE METABOLIC EFFECTS OF ADRENALECTOMY AND ADRENAL CORTEX EXTRACT ADMINISTRATION

Insofar as carbohydrate metabolism is concerned, the adrenalectomized animal is quite similar to the hypophysectomized Long, Katzin and Fry (22) found that, like the hypophysectomized rat, the fasting adrenalectomized rat or mouse cannot maintain its stores of liver and muscle glycogen The fed animal, provided it is maintained with NaCl, is not different from normal in this respect There is evidence that adrenalectomy results in a greater oxidation of glucose by the tissues although this has been denied by Russell (9) The phlorhizinized adrenalectomized animal utilizes more glucose than the normal (23) Observations on the overall R Q following adrenalectomy are conflicting some observers finding no change while others have found an elevation The adrenalectomized animal is also insulin sensitive and although the medulla may be

partly responsible, loss of the cortex appears to be the chief factor (24)

The administration of adrenal cortex extract (ACE) to normal or adrenalectomized fasting mice or rats results in a 10 to 20 fold increase in liver glycogen with very little effect on muscle glycogen When the animals are fed, there is also an increase in muscle glycogen The deposition of liver glycogen under the action of hormones of the adrenal cortex is the basis of a bioassay for steroids with respect to their action on carbohydrate metabolism (25) In addition to its effect on tissue glycogen ACE tends to raise the blood sugar level Since ACE also causes a rise in urinary nitrogen excretion it appears that the increased liver glycogen is due to formation of carbohydrate from protein sources ACE not only influences the production of carbohydrate, but it also diminishes carbohydrate oxidation Following ACE injection in rats Long Katzin and Fry (22) observed a fall in R Q from 0 86 to 0 78 although the oxygen consumption remained the same Ingle (26) has also presented evidence that ACE tends to depress the glucose tolerance of the eviscerated rat in the presence of insulin In the partially depancreatized diabetic rat ACE augments the glycosuria (22) while at the same time increasing urinary nitrogen excretion

We may again ask how much of the effects of hypophysectomy or APE administration are due to a lack of or an excess of adrenal cortical hormones? The effects of ACE on the metabolism of hypophysectomized rats are essentially the same as in normals and ACE given to the Houssay animal (hypophysectomized-depancreatized) reestablishes the diabetes But the diabetogenic action of pituitary hormones appears to require the presence of the adrenals Long Katzin and Fry (22) were unable to increase the glycosuria of partially depancreatized rats by

APE following adrenalectomy Long and Lukens (2) could not exacerbate the diabetes of depancreatized adrenalectomized cats with pituitary extracts

## THE METABOLIC EFFECTS OF THE ADRENAL C 11 OXYSTEROIDS (GLUCOCORTICOIDS)

Recent advances in the field of steroid chemistry have resulted in the isolation and identification of many compounds from the adrenal cortex The reader is referred to the several volumes of Vitamins and Hormones for details of these findings It has become clear as a result that those adrenal steroids of the type of corticosterone having an oxygen atom at carbon atom 11 possess greater potency in the liver glycogen deposition test than those steroids without C 11 oxygen such as desoxycorticosterone The 11-desoxy (without oxygen) steroids of which desoxycorticosterone is an example are chiefly responsible for the electrolyte regulatory effects of adrenal cortical extracts This differentiation between the two types of adrenal steroids is only quantitative since large doses of corticosterone may affect electrolyte balance while large amounts of desoxy corticosterone may alter carbohydrate metabolism

The recent discovery of Hench *et al* (27) of the profound effects of cortisone (11 dehydro 17 hydroxy corticosterone) in rheumatoid arthritis and allied diseases has resulted in greatly stimulated interest in the metabolic effects of the steroid hormones From the point of view of our discussion it is interesting that very large doses of cortisone of the order of 50 100 mg per day are necessary in the treatment of rheumatoid arthritis In comparison the amounts of active steroids present in ordinary adrenal cortical extracts are minute indeed It may be superfluous to point out that the concentration of hormones in an extract of a gland gives no indication of the extent to

which they can be manufactured In view of our lack of knowledge concerning the kind and amount of those steroids which can be elaborated by the gland *in vivo*, it is difficult to draw conclusions about the insulin antagonistic effects observed with adrenal extracts

With this realization in mind it may be stated that, so far, the effects of C-11 oxysteroid administration are in keeping with what we would expect from the alterations in carbohydrate metabolism following adrenalectomy or ACE injection The 11-oxysteroids will cause liver glycogen deposition, exaggeration of pre-existing diabetes, and in themselves may produce a diabetic like state (1) On the basis of experiments on the glucose tolerance of eviscerated rats Ingle (28) has concluded that the glucocorticoids depress carbohydrate utilization These effects are exactly opposite to the action of insulin

## *IN VITRO* STUDIES OF PITUITARY AND ADRENAL MODIFICATION OF THE ACTION OF INSULIN

It is a large step from a study of the physiology of insulin action in the intact animal to a study of its effects in living tissue kept outside the body *In vitro* studies eliminate many variables and afford the investigator control over the metabolic environment of the surviving tissue In the interpretation of experimental results this control brings the cause and effect relationship into sharper focus and one can be more certain that the given experimental procedures were directly responsible for the observed result This advantage is also a disadvantage for we are primarily interested in occurrences in the intact animal Yet studies of *in vitro* tissue metabolism have yielded much information concerning the mechanism of insulin action

The forthcoming discussion is concerned mainly with

*in vitro* studies of the metabolism of the rat diaphragm This organ is well suited for such investigation because it can be removed easily with little trauma, it is thin enough to allow adequate diffusion of oxygen when placed in a Warburg vessel in an atmosphere of oxygen, and the effects of insulin on this tissue are reproducible

Two approaches to the investigation of the influence of pituitary and adrenal factors on the action of insulin *in vitro* are (a) The living animal is treated with the sub stance in question, the diaphragm is removed and its response to insulin determined *in vitro* The results are then compared with normal, untreated animals (b) The diaphragm is removed from the animal and exposed *in vitro* to pituitary or adrenal hormones The results can be compared with the control hemidiaphragm which is not treated We will consider first results obtained with pro cedure (a)

## THE EFFECTS OF HYPOPHYSECTOMY AND ANTERIOR PITUITARY EXTRACT ON THE RESPONSE OF THE ISOLATED RAT DIAPHRAGM TO INSULIN

Gemmill (29) showed that insulin promotes the synthesis of extra glycogen from glucose by the isolated rat dia phragm Stadie and co-workers (30) have devised a special technique which aids in the quantitative measurement of the response of the diaphragm to insulin, and have in vestigated the effects thereon of pituitary and adrenal hormones (31)

The hypophysectomized animal is hypersensitive to insulin Bornstein and Nelson (32), and Stadie, Haugaard and Marsh (33) have found that the isolated rat diaphragm under proper conditions, likewise exhibits insulin hyper

sensitivity The ability of the normal diaphragm to syn thesize extra glycogen under the action of insulin is there fore limited by the presence of some factor, directly or indirectly of pituitary origin Since the diaphragm from adrenalectomized rats has not been shown to be hyper sensitive to insulin it is likely that the factor missing in the hypophysectomized rat diaphragm is of pituitary origin The removal of a restraining element by hypophysectomy suggests that administration of pituitary extracts to the intact animal should render the diaphragm refractory to insulin This was first found by Nelson (34) and later confirmed by Stadie *et al* (31)

## THE INSULIN ANTAGONISTIC EFFECTS OF PURIFIED GH AND ACTH

Stadie *et al* (31) in their investigations on the effects of crude APE administration found that the growth hor mone was chiefly responsible for the decreased insulin effect This was also found by Li Kalman and Evans (35) Krahl and Park (36) found that GH injection depressed the glucose uptake of the rat diaphragm and that the glucose uptake of diaphragms from hypophysectomized rats was increased The evidence derived from *in vitro* study of the metabolic effects of the GH is thus in accord with the findings obtained in the intact animal One may conclude that the growth hormone inhibits the utilization of glucose and diminishes the response to insulin

Li, Kalman and Evans (37) found that ACTH injection decreased the insulin effect on glycogen synthesis in the rat diaphragm With the exception of this observation the effects of ACTH on the response of the rat diaphragm to insulin have not been thoroughly studied

## THE EFFECTS OF ADRENALECTOMY AND ADRENAL STEROID ADMINISTRATION ON THE RESPONSE OF THE RAT DIAPHRAGM TO INSULIN

Although the adrenalectomized animal is also hyper sensitive to insulin, it has not been possible to demon strate this in the isolated diaphragm The diaphragm from adrenalectomized rats appears to be little different from normal with respect to glucose uptake, glycogen synthesis and response to insulin Villée and Hastings (38) have re ported an enhanced glucose uptake in the adrenalec tomized rat diaphragm There is a gap here in our knowl edge It may be that the insulin hypersensitivity of the intact adrenalectomized animal is due to alteration in liver metabolism with muscle playing a minor part Cheng and Sayers (39) have produced a state of insulin sensitivity in the rat by the unexpected procedure of implanation of pellets of desoxycorticosterone According to Sayers (39, 40) this may be explained by assuming that DOCA administration inhibits ACTH production by the pitui tary, *resulting in a relative deficiency of C-11* oxysteroids in the body

Administration of ACE to the intact animal produces a diminution in response to insulin according to Li, Kalman and Evans (37) Stadie *et al* have shown that injection of cortisone will inhibit the insulin effect in the isolated rat diaphragm, while ACE injection had no effect (31, 41) *To further complicate matters, cortisone* was not active in the adrenalectomized or hypophysectomized animal Since the hypophysectomized and adrenalectomized animals are deficient in steroid hormones, it is perhaps not surprising that cortisone does not have the same effect on the response to insulin as in the normal This aspect of the problem has

not yet been clarified It is possible to state however that experiments *in vitro* on the effects of the adrenal glucocorticoids corroborate in general the results of *in vivo* work If the experimental conditions are chosen correctly it can be shown that the adrenal cortical hormones oppose the action of insulin on the synthesis of glycogen by rat diaphragm

## *IN VITRO* STUDY OF THE SYNERGISTIC ACTION OF ANTERIOR PITUITARY AND ADRENAL CORTEX IN THE REGULATION OF THE RESPONSE OF THE RAT DIAPHRAGM TO INSULIN

The presence of the adrenals appears to be necessary for some of the effects of the growth hormone It is interesting to note that one must wait 12 to 24 hours for the effects of a single injection of growth hormone (31) The lack of effect of GH in the adrenalectomized animal may be related to the loss of steroids from the tissues When cortisone is given along with GH there is a depression of the insulin effect which is especially striking since neither agent alone has such action when the adrenals are absent (41) The results of cortisone and GH injection in the hypophysectomized rat are the same as in the adrenalectomized Neither agent alone depresses the insulin effect in combination there is a significant inhibition The work of Krahl and Park (36) is in accord with the concept of a synergism between the adrenal steroids and the growth hormone These workers investigated the effects of GH preparations on the glucose uptake of the rat diaphragm It was found that while GH injection depressed glucose uptake in normal rat diaphragms it did not do so in the adrenalectomized hypophysectomized animal unless lipo adrenal extract was given concurrently Furthermore the amount of GH required to inhibit glucose uptake was less

if adrenal extract was also injected The experiments of Tepperman and Tepperman (18) on the effect of growth hormone on the production of ketones by liver slices also lead us to the conclusion that the presence of the adrenal cortex is necessary for many of the effects of the growth hormone

To recapitulate, it has been demonstrated that the hormone responsible at least in part for the insulin antagonistic effects of crude anterior pituitary extracts is the growth hormone In order to exert this action, it requires the presence of a certain amount of C 11 oxysteroids in the tissues

## SOME OBSERVATIONS ON THE TREATMENT *IN VITRO* OF RAT DIAPHRAGM FROM NORMAL RATS WITH ADRENAL AND PITUITARY SUBSTANCES

Effects of pituitary and adrenal hormones have been obtained entirely outside the body by treating diaphragms from normal rats with these substances It is well to consider the difficulties of such experiments Inhibitory effects may easily be encountered if the tissue is damaged in some way by the substance to be tested Furthermore, it is often hard to determine whether the observed effects are a specific property of the substance tested or whether the entire class of compounds to which the test substance belongs will act in a similar manner This is the chief difficulty with experiments involving the steroids, for it has been found (42) that the presence of small amounts of almost any steroid substance in the medium in which a diaphragm is suspended will bring about glycogenolysis, or at least prevent the tissue from actively synthesizing glycogen With present techniques, therefore, it is impossible to assert that the C-11 oxysteroids possess a *unique* ability

to produce glycogenolysis or to oppose the action of insulin Naturally this difficulty is not present when the animal is treated with the steroid in question prior to removal of the diaphragm

In spite of the obstacles mentioned considerable success has been achieved in demonstrating that anterior pituitary extracts *in vitro* will alter the response of the diaphragm to insulin Ottaway and Smith (43) reported that treatment of the diaphragm with anterior pituitary extract for 30 minutes abolished the insulin effect on glucose uptake and glycogen synthesis during a subsequent period of incubation in medium containing glucose but no pituitary extract This has been confirmed by Stadie *et al* (31) who found that after pretreatment with APE the tissue could be *washed* before adding insulin without losing the inhibitory substance

The factor does not appear to be growth hormone since pre equilibration of the diaphragm with this substance (44) has been shown to cause an increase in glucose uptake by the diaphragm an effect quite different from that observed with crude pituitary extract Although some partially purified pituitary preparations have exhibited an anti insulin effect when tested in the rat diaphragm it is not yet possible to identify this substance with any of the known pituitary principles Whatever the identity of the active factor may be further studies of the *in vitro* action of pituitary extracts will aid in understanding the way in which pituitary hormones and insulin regulate cellular metabolism

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## CHAPTER VIII

# SOME CLINICAL ASPECTS OF THE ACTION OF INSULIN

THE EXPERIMENTS inadvertently performed on humans offer interesting examples of alterations in the response of individuals to insulin. Probably the glucose tolerance test is the most frequently employed clinical test having a direct bearing on the mechanism of insulin action. A typical normal and "diabetic" curve are shown in Figure 1. A "diabetic" curve may signify a lack of insulin, but does not indicate whether this is an absolute or a relative deficiency. We do not yet know the etiology of diabetes, and it is probable that factors other than the amount of insulin secreted by the pancreas are involved. The regulation of the concentration of glucose in the blood is a complex equilibrium, involving the mobilization of liver glycogen, the utilization of glucose by the tissues, and the endocrine secretions. This focuses attention on those diseases, chiefly of the endocrine system, associated with an abnormal response to insulin and a derangement of carbohydrate metabolism.

## THE INSULIN TOLERANCE TEST AND THE GLUCOSE INSULIN TOLERANCE TEST

An altered response to the administration of insulin—either sensitivity or resistance to its effects—occurs frequently in disease of the endocrine glands. The insulin tolerance test, introduced by Himsworth (1), measures the effect of a known quantity of insulin on the blood sugar level. Modifications of the insulin tolerance test, such as

the glucose insulin test have the advantage of avoiding undesirable hypoglycemia Such a test has been described by Engel and Scott (2) and the normal curve obtained by

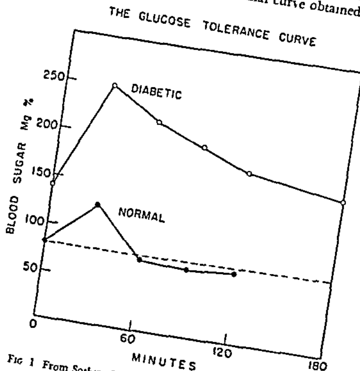



Fig 1 From Soskin S *J Clin Endocrinology* 4 75 (1944)

these workers is shown in Figure 2 It is apparent from this curve that when glucose is given during the period of hypoglycemia following the injection of insulin marked hyperglycemia occurs This rebound phenomenon was recently described by Somogyi (3)

An example of the insulin resistant response in the

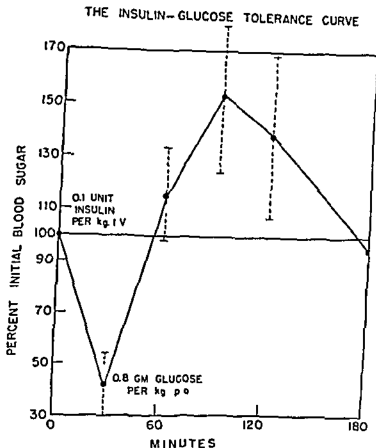



*Fig 2 From Engel F L. and Scott J L. J Clin Investigation,* 29 151 (1950)

*glucose insulin tolerance test is shown in Figure 3 Here* the hypoglycemic effect of insulin is lacking, and the high level of blood sugar returns slowly towards normal Resistance or antagonism to the action of insulin resulting in a curve of this kind might be expected in disease conditions associated with the secretion of substances whose

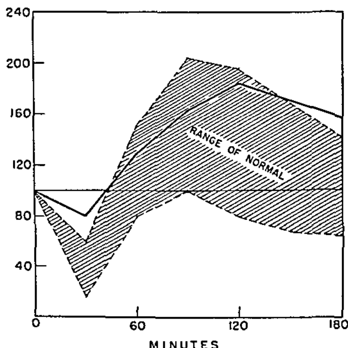



FIG 3 From Engel F L, and Scott J L *J Cl n Invest gat on* 29 151 (1950)

action is opposed to that of insulin—notably adrenal or pituitary hormones Some of these diseases are

1 Hyperpituitarism occurring in (a) gigantism or active acromegaly due to eosinophil cell adenoma diffuse hyperplasia of the eosinophil cells or to unknown causes or (b) Cushing s syndrome with basophil adenoma and/or adrenal cortical adenoma

2 Hyperadrenocorticism, occurring in (a) adrenal cortical adenoma or carcinoma, or (b) diffuse adrenal cortical hyperplasia

3 Diabetes mellitus when associated with insulin resistance

4 Some cases of thyrotoxicosis

In the hyperpituitary individual, excessive secretion of the growth hormone by the tumor cells may be responsible for counteracting the effect of insulin Although the eosinophil cells appear to secrete growth hormone, not all active pituitary tumors arise from them Hypersecretion of ACTH by a pituitary tumor may lead to the release of insulin opposing steroids from the adrenals The adrenal cortical adenomas commonly secrete an excess of C-11 oxysteroids which would provide an adequate explanation for the associated resistance to insulin Insulin resistance in diabetes will be discussed later in this section True allergy to insulin may account for some of these cases In thyrotoxicosis the general increase in metabolic rate may render the subject relatively insensitive to a dose of insulin ordinarily effective For a discussion of the role of the thyroid with respect to diabetes the reader is referred to that of Houssay (4)

In Figure 4 we see an illustration of the opposite abnormal response to insulin hypersensitivity There is an exaggeration of the hypoglycemic reaction and a delayed return of the depressed blood sugar level to normal The hyperglycemic response to glucose given during the hypoglycemic period does not occur Conditions in which insulin sensitivity may occur include

1 Hypopituitarism, as in Simmonds disease, due to destruction of the pituitary by embolism or thrombosis of its blood supply or by pressure from non functioning tumors such as the craniopharyngeal pouch group

(2) Hypoadrenocorticism or Addison's disease caused by adrenal loss from tuberculosis atrophy etc

3 Some cases of hyperinsulinism with islet cell tumor

4 Some cases of hypothyroidism

Sensitivity to insulin in these diseases may be ascribed to the lack of the counter balancing secretions of the adrenal

INSULIN—GLUCOSE TOLERANCE CURVE
SHOWING HYPERSENSITIVITY TO INSULIN

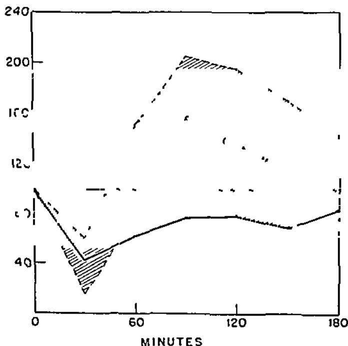



Fig 4 From Engel F L and Scott J L *J Clin Investigation* *29* 151 (1950)

or pituitary glands This is particularly true in panhypopituitarism where the adrenals are atrophic because of the loss of ACTH Lack of opposing thyroid hormone may explain the occasional case of myxedema which manifests sensitivity to insulin, although the nature of the antagonism between the thyroid and insulin is not well understood at present

Although abnormal responses of the kind depicted in Figures 3 and 4 are frequently observed, the insulin and glucose insulin tolerance tests are of limited diagnostic value because of the large number of exceptions to the expected result Furthermore, the interpretation of the results of the test itself is subject to certain errors If the glucose is administered orally, changes in the rate of intestinal absorption, as sometimes happens in myxedema, will affect the result In the presence of severe liver disease impairment of the glycogenolytic process rather than sensitivity to insulin may result in considerable lability of the blood sugar concentration A striking example of this occurs in Von Gierke s disease (glycogen storage disease) in which the patient is often hypoglycemic since the ability to mobilize liver glycogen has been impaired Finally, the variation in response of normal individuals to insulin makes it difficult to achieve normal limits sufficiently narrow for diagnostic purposes There is no doubt however that the insulin tolerance tests in human subjects yield results in conformity with present knowledge concerning the action of insulin which has been derived from studies in experimental animals

## THE HYPERGLYCEMIC GLYCOGENOLYTIC FACTOR

In discussing the subject of the effect of insulin on the blood sugar concentration it should be noted that most

commercial preparations of insulin contain the hyper glycemic glycogenolytic factor (HGF) as an impurity Ad ministration of this substance will result in a temporary hyperglycemia due to liver glycogenolysis It is too early to assess the clinical importance of the HGF Lesions of the α cells of the pancreas which contain HGF have been described by McQuarrie (8) in certain cases of spontaneous hypoglycemia If the HGF counteracts the action of insulin by raising the blood sugar disease of the α cells may result in spontaneous hypoglycemia Evidence for the physiological importance of HGF has been obtained by Bornstein Reid and Young (9) who found that growth hormone stimulated the production of HGF by the pancreas

## THE PROBLEM OF INSULIN RESISTANCE

The insulin requirements of patients with diabetes mellitus are variable In many persons the disease may be controlled by diet alone When a diabetic who has been using 20 units of insulin daily suddenly requires 200 units to remain free from glycosuria he has developed resistance to insulin It is difficult however to impose arbitrary limits of insulin dosage above which resistance may be said to exist Cases have been reported in which several thousand units were given every day (5) For the purposes of this discussion we may divide the cases of insulin resistance into those which are mild or severe arbitrarily including in the latter category persons requiring 300 units or more of insulin per day The underlying mechanism of insulin resistance may be the same in all cases

It has been thought by some observers that cases of insulin resistance in which extremely large doses are necessary are associated with allergy to insulin Franklin and Lowell (6) have recently shown that patients resistant to

insulin prepared from beef or pork sources will respond to insulin prepared from human pancreas These same authors have also experimentally produced allergy and resistance to insulin in rabbits (7) One explanation for some cases of insulin resistance, therefore, is that anti bodies to insulin, present in the blood, combine with the injected insulin, rendering it inactive Lowell (10) has shown that when insulin is mixed with serum obtained from a severe case of resistance, the insulin loses the ability to produce convulsions in mice This successful demon stration of insulin neutralizing substances in the serum of resistant patients suggests that the underlying mechanism actually was an inactivation of the injected insulin rather than interference with its action in the tissues This does not prove, however, that the substance in serum is an anti body *In fact, in Lowell s patient, the allergic phenomena* seemed to vary independently of the degree of resistance to insulin Whatever the nature of the insulin neutralizing substance in serum, it would be important to establish the fact that resistance to insulin is associated with its inactiva tion by serum

In an attempt to study the nature of the insulin inactivating properties of serum, we have performed some experiments employing the rat diaphragm *in vitro* as a test object (11) In these experiments, summarized in Table VI, the response of the rat diaphragm to insulin as meas ured by its effect in promoting glycogen synthesis was de termined when the insulin had been mixed with serum When the rat diaphragm was exposed to insulin alone, at a concentration of 0 1 unit/ml, it synthesized extra glycogen amounting to 8 µM per Gm But if the dia phragm was placed in a solution containing serum as well as insulin, the subsequent insulin effect was only 5 6 This indicates that even normal serum has in some degree the

property of combining with or inactivating insulin in such a way as to lower its effective concentration This con clusion follows from the fact that the amount of extra glycogen synthesized by the diaphragm is a function of the concentration of insulin in the solution to which it has been exposed (12) It can be seen from Table VI that serum from non resistant diabetics is not different from normal in its ability to lower the effective concentration of insulin Serum from three cases of severe insulin resistance, how ever, completely abolished the insulin effect (the small

TABLE VI

THE EFFECT OF SERUM FROM NORMAL AND INSULIN RESISTANT INDIVIDUALS ON THE COMBINATION OF INSULIN WITH RAT DIAPHRAGM

Serum diluted 1 1 with medium and incubated five minutes with 0 1 unit of insulin per ml Diaphragm then added for one minute and allowed to synthesize glycogen for 90 minutes

| *Source of serum* | *Insulin requirements units per day* | *Insulin effect on glycogen synthesis as µM glucose/Gm* | *No of experiments* |
|---|---|---|---|
| No serum | — | 8 3±0 52 | 75 |
| Normal persons | 0 | 5 6±0 92 | 11 |
| Mild diabetics | 0–20 | 5 3±1 05 | 6 |
| Insulin Resistant, Severe | | | |
| Case I | 800 | 0 6±0 67 | 5 |
| Case II | 600 | 0 5±0 98 | 5 |
| Case III (a) | 300 | 4 2±0 98 | 5 |
| (b) | 170 | 4 0±1 75 | 4 |
| Insulin Resistant Mild | | | |
| Case IV (a) | 200 | 3 2±1 09 | 5 |
| (b) | 20 | 7 0±1 46 | 4 |
| Case V | 150 | 4 8±0 71 | 4 |
| Case VI | 150 | 5 1±2 25 | 3 |

positive values shown are not significantly different from zero) Even more interesting is the fact that the extent of inactivation of the insulin in the presence of serum was roughly proportional to the insulin requirements as shown in Figure 5

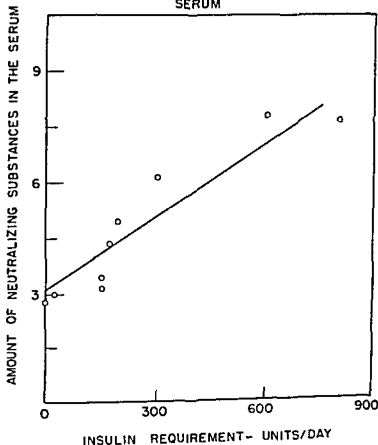



FIGURE 5

At present, we may only speculate about the nature of the substance present in serum which seems to be associated with resistance to insulin The possibility that it is an antibody protein has already been discussed Bornstein and Trewhella (13) reported some interesting experiments in which the serum from normal diabetic and insulin resistant patients was assayed for its insulin content This was made possible by the use of an adrenal-demedullated alloxan diabetic, hypophysectomized rat which is extremely sensitive to insulin These workers found no insulin in the serum of the insulin resistant cases However, many of the non resistant diabetic cases likewise had no detectable insulin in their serum Perhaps the insulin may be present but combined with some component of serum since we have shown that insulin reacts with serum to some extent even in normal individuals The insulinase described by Mirsky and Broh Kahn (14) an insulin inactivating material present in liver extracts and in some other tissues may be responsible for excessive destruction of insulin in resistant persons There is no evidence at present for this idea The nature and significance of the insulin inactivating properties of human serum remains to be established in future work

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# CHAPTER IX

# THE MECHANISM OF ACTION OF INSULIN

IN THE SEARCH for a locus of insulin action, recourse has been taken to the study of tissue metabolism *in vitro* using tissue slices, minces, or extracts With such preparations it is possible to study the effect of insulin on an isolated reaction or on the metabolism of one tissue, and in contrast to experiments with whole animals it is somewhat easier to interpret the results obtained

For many years it has been the hope of those studying the problem that insulin might be found to inhibit or accelerate a chemical reaction catalyzed by a soluble enzyme Attempts to demonstrate a direct effect of insulin on the activity of a purified enzyme have been unsuccessful On the contrary, it has been found that the more intact or organized the tissue structure, the easier it has been to demonstrate an action of insulin

Extensive work during the last 15 years has resulted in the demonstration of effects of insulin *in vitro* on glucose and glycogen metabolism on reactions involving oxygen uptake, and on fat and protein metabolism

## EFFECT OF INSULIN ON GLUCOSE AND GLYCOGEN METABOLISM

Cruickshank and Shrivastava (1) using a heart lung preparation demonstrated that insulin increased the utilization of glucose by the diabetic and by the normal cat heart Cruickshank and Startup in a later work (2) studied glucose uptake oxygen consumption and carbon dioxide production in the isolated heart lung preparation

from normal and diabetic cats In the diabetic heart they found practically no glucose utilization and an RQ close to 0 70, indicating a predominance of fat metabolism Following the addition of insulin to the perfusing fluid a marked increase in the utilization of glucose and a rise in the RQ to almost 1 00 was observed The authors concluded that the primary defect in the diabetic heart was a failure to utilize carbohydrate The diabetic heart contains a large amount of glycogen and insulin was found to have little effect on glycogen synthesis

The most reproducible effect of insulin *in vitro* is that of increasing the glucose utilization and glycogen production by the isolated rat diaphragm This property of insulin was first discovered by Gemmill (3) Stadie and Zapp (4) studied in detail the effect of insulin in diaphragms *from normal rats and defined the conditions under which* an optimal response to insulin was obtained The isolated diaphragm has now been used extensively for the study of the action of insulin and pituitary and adrenal cortical hormones on carbohydrate metabolism

It is interesting that although the glucose uptake and *glycogen synthesis of the diaphragm are increased by* insulin, there is no significant effect on the oxygen uptake *or RQ Haugaard Marsh and Stadie (5) observed,* however, that in the presence of insulin there was a decrease *in the* acid labile phosphate (presumably mostly ATP) and a corresponding increase in the difficulty hydrolyzable phosphate esters (such as the hexose phosphates)

Work on the glucose uptake and glycogen synthesis of the liver has not been similarly successful Glycogen formation *in vitro* by liver slices was first demonstrated by Cross and Holmes (6) and by Ostern Herbert and Holmes (7) They found that a high concentration of glucose in the medium was necessary and only comparatively low

rates of glycogen synthesis were observed No reproducible effect of insulin was found Crandall (8) also studied the synthesis of glycogen by liver slices He demonstrated formation of glycogen by liver slices equilibrated in a bicarbonate medium at a high pH and at a high concentration of glucose There was a significant positive correlation between the synthesis of glycogen and the RQ and oxygen uptake of the liver slices No experiments with insulin were reported In view of the ease with which effects of insulin can be demonstrated in the diaphragm it is curious that insulin has so little effect in similar experiments with liver slices There are several factors which might be responsible for the difficulty in demonstrating effects of insulin on carbohydrate metabolism in liver slices In contrast to the diaphragm which can be removed with very little trauma there is necessarily considerable damage involved in the preparation of tissue slices It is also known that liver is especially rich in the insulin inactivating system described by Broh Kahn and Mirsky (9) Finally the glycogenolytic factor present in many insulin preparations causes a production of glucose from glycogen in liver slices (10)

Since insulin undoubtedly influences carbohydrate metabolism in the liver of the intact organism and since the liver is a key organ in the metabolic transformations performed in the body it appears worth while to search for conditions under which insulin effects can be conclusively demonstrated in liver preparations It may be recalled that *in vitro* effects of insulin on fat metabolism in liver slices have been found In addition Kaplan and Greenberg (11) have demonstrated a greater turnover of ATP in the liver after addition of insulin

*In vitro* synthesis of glycogen from glucose has been demonstrated in heart slices (12) It is interesting that a

medium practically devoid of electrolytes was found to offer the best conditions for glycogen synthesis The glycogen synthesis was only slightly influenced by insulin The carbohydrate metabolism of the heart differs from that of the diaphragm It is well known that the level of heart glycogen increases above normal in the diabetic and is apparently related to the level of blood sugar rather than to the supply of insulin

## EFFECTS OF INSULIN ON OXIDATIVE REACTIONS

The first well established effect of insulin *in vitro* was its effect on the oxygen uptake of pigeon breast muscle Krebs and Eggleston (13) equilibrated pigeon breast muscle minces for several hours and found that the addition of insulin accelerated the rate of oxygen uptake The effect was not an immediate one but constituted a prevention of the usual decline of the rate of oxygen uptake These findings have been amply confirmed (14 15 16)

This action of insulin long remained an isolated observation to which little attention was paid The argument was used that these experiments were of no fundamental importance since the effect of insulin could only be demonstrated in one tissue preparation and that from a non mammalian species Rice and Evans (17) later restudied the effect of insulin on the respiration of pigeon breast muscle mince and found that when the mince was equilibrated in the presence of pyruvate both oxygen uptake and pyruvate utilization were increased An increased pyruvate utilization was also found in the presence of malonate with oxaloacetate and pyruvate as substrates The authors concluded that insulin in some way accelerated one of the reactions involved in the oxidation of pyruvate

Stadie, Haugaard and Perlmutter (18) confirmed the essential findings of Rice and Evans. An increased oxygen uptake in the presence of insulin was observed with slices or minces of pigeon breast muscle but not with extracts of this tissue. However, no effect of insulin was found in similar experiments with rat muscle minces.

It has become clear that the effect of insulin on oxygen uptake in pigeon muscle is not an isolated phenomenon, but that other tissues respond to insulin in a similar manner. In three other tissues an effect of insulin on respiratory metabolism has been demonstrated. Fisher, Hall and Stern (19) reported an increase in the oxygen uptake of frog muscle strips equilibrated *in vitro*. Balmain, Foley and French (20) showed that insulin raised the oxygen uptake and RQ of slices of mammary glands from lactating rats. The slices were respiring in a phosphate bicarbonate medium containing acetate and glucose as substrates. The increase in RQ indicates that insulin accelerated the transformation of acetate and glucose to fat in this system. Later this conclusion was confirmed by the direct demonstration of an increased incorporation of $C^{14}$ acetate into higher fatty acids (21).

Haugaard and Marsh (22) have shown that insulin increases the oxygen uptake of minces or strips of rat adipose tissue. This effect was not found in the absence of added substrate but could be demonstrated in the presence of many different substances. It was equally well obtained in the presence of lactate, pyruvate, succinate or glucose. The insulin effect on oxygen uptake was evident at the very beginning of the equilibration, implying an immediate action of insulin on some process involved in determining the rate of oxygen uptake. As in other cases insulin was without effect when the tissue was broken up by homogenization. The strips or minces exhibited an RQ well

over 1 00 indicating fat formation The homogenate al though respiring quite actively, produced very little carbon dioxide, with values of RQ less than 0 5 An old observation of Cori (23) should be mentioned in this con nection He found that insulin increased the oxygen up-take of rat liver homogenates under certain conditions However most liver homogenates were not affected by added insulin and it was not possible to find conditions under which this effect of insulin could be made reproducible Finally, Banga Ochoa and Peters (24) reported that insulin increased the utilization of pyruvate by slices of pigeon brain

The observations discussed in this section indicate that insulin may be involved in some way with the regulation of reactions concerned with oxidative metabolism

## EFFECT OF INSULIN ON FAT METABOLISM

There are many indications that insulin influences the metabolism of fat As we have mentioned earlier, the ex periments of Stetten and Boxer have demonstrated con clusively that one of the main defects in diabetes is an inability of the organism to synthesize fat Recently these results have been corroborated by experiments *in vitro* Liver slices from diabetic rats were found to have a greatly diminished ability to synthesize fat from acetate (25) or glucose (26) Bloch and Kramer (27) showed that in the presence of pyruvate insulin increased the incorporation of radioactive acetate into fatty acids by rat liver slices Brady and Gurin (28) confirmed these findings but found that the presence of pyruvate was not necessary to demon strate the insulin effect. The synthesis of fat by cat liver slices was also increased by insulin (29) Liver slices from alloxanized rats or depancreatized cats had almost com pletely lost their ability to synthesize fat from acetate

Curiously enough insulin had little effect in restoring fat synthesis to normal in liver slices from diabetic animals Recently, it has been found that the ability of liver slices to synthesize fatty acids from acetate is directly related to the glycogen content (30) Furthermore, epinephrine and the hyperglycemic glycogenolytic factor, which both cause glycogenolysis in liver slices diminish the rate of fat synthesis (31) These hormones, therefore have an action opposite to that of insulin in this system

## PROPOSED THEORIES OF THE ACTION OF INSULIN

The diversity of the metabolic processes affected is a remarkable characteristic of the effects of insulin observed *in vitro* Carbohydrate fat and protein metabolism have all been found to be under the regulatory influence of insulin How are such widely different actions of insulin brought about? It is hardly likely that insulin has a direct action on a great number of entirely different chemical reactions An effect of insulin on one important reaction may cause such an imbalance in tissue metabolism that many different metabolic reactions are indirectly affected The variety of the effects of insulin observed may also be explained if it is postulated that insulin regulates one single type of metabolic reaction common to the metabolism of many different substrates

## THE HEXOKINASE THEORY OF INSULIN ACTION

The phosphorylation of glucose to glucose 6-phosphate by ATP is the first step in the metabolism of glucose The enzyme hexokinase catalyzes this reaction The hexokinase reaction is a logical one to investigate as the site of insulin action, since facilitation of the initial metabolic reaction

of glucose would explain the acceleration of all subsequent reactions, including glycogen synthesis The increased glucose uptake of the rat diaphragm brought about by insulin can only be explained by assuming that the rate of the hexokinase reaction is increased However, it is impossible to decide from this fact alone whether the change is caused by a direct effect of insulin on the hexokinase enzyme or by an increased availability of ATP

Attempts to demonstrate a *direct* stimulatory effect of insulin on the hexokinase reaction in tissue extracts have been unsuccessful In 1945, Price, Cori and Colowick (32) stated in a preliminary report that the hexokinase activity of rat muscle extracts *was inhibited by the addition of* anterior pituitary extract (APE), and that this inhibition was relieved by insulin Later, Colowick, Cori and Slein (33) made the following observations

(1) *In 50% of the experiments the addition of* adrenal cortex extract (ACE) to muscle extracts from alloxan diabetic rats resulted in an inhibition of the hexokinase activity When such inhibition occurred, it was abolished *by the addition of insulin to the system*

(2) The activity of partially purified beef brain hexokinase preparations was diminished by the *in vitro* addition of certain preparations of APE plus ACE *This inhibition was likewise counteracted by insulin*

These experiments recall the Houssay (hypophysectomized depancreatized) and the Long Lukens (adrenalectomized-depancreatized) experimental diabetic states In *these animals there is an amelioration of the diabetes due* to the loss of insulin antagonistic substances of pituitary or adrenal origin In the experiments of Colowick, Cori and Slein inhibitory effects of ACE and APE on the *hexokinase reaction were removed by the addition of* insulin These observations seemed to indicate that insulin

altered the rate of the hexokinase reaction in the body by overcoming the physiological inhibition of adrenal and pituitary factors This represents the hexokinase theory of insulin action as expressed by Cori (23) It should be noted that the original investigators themselves did not claim that this represents the only site of insulin action

The importance of the observations of Cori and his collaborators if firmly established cannot be overestimated They constitute for the first time a demonstration of an interrelationship between a hormone and an enzyme in a cell free reaction system However the difficulties in obtaining reproducible effects of insulin in tissue extracts have been so great that the original observations have not been adequately confirmed

Broh Kahn and Mirsky (34) were not able to demonstrate an effect of insulin on the hexokinase reaction in muscle extracts from diabetic rats However in a few experiments they did demonstrate a decrease in the hexokinase activity of brain hexokinase preparations and a reversal of the inhibition by insulin Extracts of rat spleen were also found to cause a decrease in the rate of the hexokinase reaction Stadie and Haugaard (35) in their study of the hexokinase reaction in muscle extracts of normal and alloxanized rats did not find any difference in reaction rate between these two groups Nor were they able to demonstrate a lowering of the hexokinase activity by the addition of adrenal cortical extract to the system No increase in the rate of reaction followed the addition of insulin Stadie Haugaard and Hills (36) also studied the hexokinase reaction in muscle extracts from depancreatized cats They were not able to demonstrate any effect of adrenal cortical extract or insulin in these experiments

Reid Smith and Young (37) in a preliminary report

stated that they had confirmed the essential findings of Cori *et al*, *including* the reversal of pituitary hexokinase inhibition There was no apparent relation between the diabetogenic activity of pituitary preparations and their ability to inhibit the hexokinase reaction In a later report from the same laboratory Smith (38) reported studies on the effect of insulin on the hexokinase activity of muscle extract from alloxanized rats Only in one extract out of 12 *was it possible to demonstrate an effect of insulin*

Christensen, Plimpton and Ball (39) studied the hexokinase activity of hemolyzed red blood cells from the rat The rate of reaction was found to be the same in preparations from normal hypophysectomized and alloxan diabetic rats

If the validity of the experiments of Cori and co-workers is *accepted the hexokinase theory explains* many of the observed relationships between the metabolic effects of insulin on the one hand and adrenal and pituitary hormones on the other It does not explain the enhanced effect of insulin in the hypophysectomized animal an objection noted by the authors themselves It is also difficult to understand how an effect of insulin on the hexokinase reaction alone could explain the numerous manifestations of insulin action It would seem necessary to postulate additional loci of insulin action Since insulin is certainly active in the absence of other hormones it is difficult to explain why *the hexokinase reaction is not affected* by insulin in normal muscle extracts

Support for the hexokinase theory is found in the experiments of Chaikoff *et al* (40) in which fructose but not glucose feeding improved the subsequent ability of liver slices from diabetic rats to synthesize fat from acetate However, its seems fair to conclude that at present the hexokinase theory of insulin action cannot be accepted unless further experimental evidence is produced

## EFFECT OF INSULIN ON OXIDATIVE PHOSPHORYLATION

The hexokinase hypothesis of insulin action concerns itself with the possible role of insulin in accelerating the initial reaction by which glucose is metabolized—its phosphorylation to glucose-6-phosphate Another hypothesis which recently has been put to the experimental test involves the possibility that insulin accelerates or increases the efficiency of the reactions by which inorganic phosphate is incorporated into high energy phosphate compounds Although the detailed reactions involved in the production of high energy phosphate compounds are not known it has been conclusively demonstrated that the energy needed for the production of high energy phosphate is obtained from reactions involved in the transfer of electrons from substrate to oxygen through the different respiratory enzymes

Such a hypothesis would explain most of the effects of insulin now known For example the effect of insulin on the synthesis of glycogen from glucose in rat diaphragm may be explained by assuming an increase in the efficiency of the coupling between phosphorylation and oxidation of metabolic intermediates so that a greater number of high energy phosphate bonds are produced per molecule of substrate oxidized The effects of insulin on fat and protein synthetic reactions might likewise reflect an increased synthesis of high energy phosphate bonds

There are many indications from experiments with intact animals and tissue preparations *in vitro* that insulin is concerned with the intermediary metabolism of phosphorylated compounds The oxidative phosphorylation hypothesis was stimulated by the findings of Sacks (41) and Kaplan and Greenberg (12) that insulin increases the turnover rates of high energy phosphate compounds such as ATP The subject of the relation of insulin to phosphate

metabolism has been reviewed critically by Stadie (43)

Haugaard Marsh and Stadie (44) found that insulin produced changes in the relative concentrations of the phosphorylated intermediates in the rat diaphragm during glycogen synthesis which were in keeping with the concept that insulin increases the turnover rate of high energy phosphate Charalampous and Hegsted (45) found that alloxanized rats had a diminished ability to acetylate para aminobenzoic acid which they attributed to a lack of available ATP

Polis and co-workers (46) studied the formation of esterified phosphate in particulate preparations of rat liver The particulate fractions were prepared by centrifugation and resuspension of the precipitates Glucose was the phosphate acceptor and yeast hexokinase was added in excess in order to prevent the hexokinase reaction from becoming the limiting factor in the system Glutamate was added as substrate It was found that insulin added to such a system caused an increased oxygen uptake and an increased formation of ester phosphate Goranson (47) found similar effects in rat brain homogenates using creatine as a phosphate acceptor He also observed that the rate of phosphorylation was lower than normal in brain homogenates from alloxanized rats The effects of insulin in these studies were not very large and not easily reproducible Polis found that many liver preparations did not respond to insulin although they apparently were prepared in exactly the same way as preparations which responded well

Although these experiments indicate that insulin may be involved in reactions involving the transfer of phosphate concomitantly linked to oxidative reactions they do not give a conclusive answer to the question of what specific reactions are concerned It is also possible that the effect of in

sulin in these systems may be on reactions involved in dephosphorylation rather than in phosphorylation For example, Drabkin and Marsh (48) have found an increase in the phosphatase activities of livers from alloxan-diabetic rats

The "oxidative phosphorylation" hypothesis of insulin action is in our opinion a promising one and should be explored further If it should be established beyond doubt that insulin is concerned with the production of high energy phosphate bonds the final clarification of the precise mode of action of insulin will again have to await the result of future studies on the mechanism by which oxidative processes give rise to the production of high energy phosphate bonds

An important mechanism in the utilization of high energy phosphate bonds is the formation of acetyl-coenzyme A It would be important to investigate the possibility that insulin is concerned with the regulation of reactions involving coenzyme A

It is now realized that oxidative phosphorylations take place in subcellular particulate components such as mitochondria Further investigations of the relation between structure and function of these cellular constituents may prove important in the understanding of insulin action

## THE PERMEABILITY THEORY OF INSULIN ACTION

The action of insulin in promoting carbohydrate utilization may be explained in still another manner It is possible that insulin may be concerned with the rate of transfer of metabolites such as glucose across the cell membrane Were it to be assumed that insulin in some way facilitates the entry of glucose into the cell through some effect on cellular permeability, the increased utilization of

glucose would be satisfactorily explained Levine *et al* (49) have attempted to *show that insulin does indeed* accelerate the entry of a metabolite into the cell The experimental results obtained were as follows it was found that if galac tose was administered to the eviscerated, nephrectomized dog, the blood level decreased rapidly and finally became stationary, if in addition insulin was added the final con centration of galactose was much lower than in the absence of insulin Insulin apparently *had caused a transfer* of galactose from the blood to the tissues A clear formulation of the Permeability' theory of insulin action has been given by Levine *et al* (49) 'Insulin acts upon the cell membranes of certain tissues (skeletal muscles, etc) in such a manner that the transfer of hexoses (and perhaps other substances) from the extracellular fluid into the cell is facilitated The intracellular fate of the hexoses depends upon the availability of metabolic systems for their trans formation In the case of galactose no further changes occur In the case of glucose, dissimilation glycogen stor age and transformation to fat are secondarily stimulated *by the rapidity of its entry into the cell*

As yet, very little work has been done which has a direct bearing on this theory of insulin action The experiments of Levine *et al* have not yet been repeated in other labora tories Should their hypothesis ultimately prove to be the correct one, *the question of the mechanism by which* insulin alters cell permeability would still be unanswered Insulin may increase permeability by affecting metabolic processes in or on the cell membrane which are involved in the entry of metabolites into the cell

Reflections on the question *of the permeability of* the cell to substances such as glucose and galactose lead one to the problem of how insulin itself enters or combines

with the cell This is a problem which the authors have been much interested in and we shall conclude this monograph with some considerations of this aspect of insulin action

## THE PROBLEM OF THE PENETRATION OF INSULIN INTO THE CELL

Ordinarily, cells are impermeable to protein If this were not the case the vital cellular proteins would be lost How is the entrance of insulin into the cell explained? One possible explanation is that specific receptors, capable of combining with insulin are present at certain cellular locations After combining with the cell, insulin is then capable of exerting its metabolic action either at the site of combination or after having been transported further into the cell Until the recent work of Stadie and coworkers (50), there was no evidence to show that cells may possess an affinity for insulin These workers studied the action of insulin on the metabolism of the rat diaphragm and came to the conclusion that a chemical combination of insulin with some cell constituent was an important part of the mechanism of action of insulin in this tissue

## THE CHEMICAL COMBINATION OF INSULIN WITH MUSCLE

The effect of insulin in promoting glucose uptake and glycogen synthesis of the isolated rat diaphragm, discovered by Gemmill, has been referred to several times. The essential finding of Stadie *et al* (50) was the demonstration that rat diaphragm, placed in a solution of insulin for a very short period of time, and then washed thoroughly, subsequently exhibited its characteristic effect in promoting the uptake of glucose and the synthesis of gl

from glucose The phenomenon was interpreted to mean that during the short equilibration insulin attaches itself firmly to the diaphragm, and in this bound state exerts its usual metabolic effect In support of this conclusion are the following observations

(1) The reaction may be demonstrated in as little as 10 seconds and reaches a maximum in about one minute

(2) The process is not easily reversible The bound insulin does not wash out, as one would expect if there were no specific mechanism for holding it to the cell

(3) The effect of insulin in promoting glycogen synthesis during the 90 minute incubation period depends on the concentration of insulin, the time of exposure of insulin the temperature, and the concentration of glucose (51)

With this technique it has been possible to study the action of insulin on metabolism in a quantitative way *When the concentration of insulin during the pre-equilibration period is low, insulin is the limiting factor in subsequent synthesis of glycogen, since increasing the concentration of insulin causes more glycogen to be formed* On the other hand the ability of the diaphragm to synthesize glycogen is limited by the concentration of glucose in the medium This is particularly well brought out in the experiments in which the diaphragms were exposed to a high concentration of insulin In these experiments there was a greatly increased insulin effect when the concentration of glucose in the medium was raised (Fig 6)

Recently the phenomenon of binding of insulin by tissue has been studied with the aid of radioactive insulin (52) Two types of labeled insulin have been used insulin in which the aliphatic hydroxyl groups have been esterified with radioactive sulfate, and iodinated insulin Using such preparations the actual amounts of insulin combining

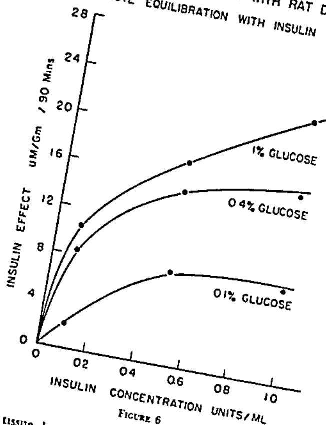



FIGURE 6

with tissue have been determined and the binding of insulin has been studied in a quantitative manner

Figure 7 shows the results of experiments in which diaphragms previously exposed for one minute to radioactive insulin at different concentrations have been

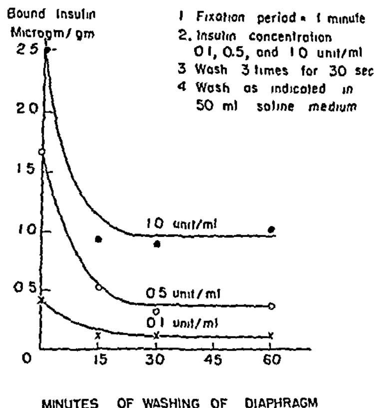


Figure 7

washed for various periods of time It is seen that a certain amount of insulin is easily removed, but the remainder resists the effect of washing The quantity of firmly bound insulin is a direct function of the concentration of insulin during the *preliminary one minute equilibration* These

experiments provide convincing evidence that insulin is able to combine firmly with tissue

Further evidence was obtained by experiments in which diaphragms were equilibrated for long periods of time in solutions containing low concentrations of insulin It was

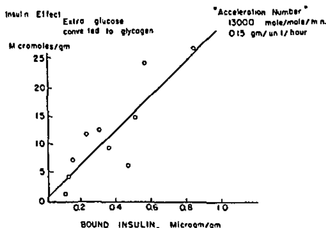


FIGURE 8

found that the diaphragm was able to remove insulin from solution and that at the end of 90 minutes equilibration the concentration of insulin in the diaphragm was greater than that in the medium

With labeled insulin it has been possible to relate the quantity of bound insulin to its biological effect Experiments of this kind are illustrated in Figure 8 Rat diaphragms were exposed to different concentrations of radioactive insulin sulfate, washed and then equilibrated in medium containing glucose The insulin effect on glycogen

synthesis and the final concentration of bound insulin in the tissue were than determined From the data it was calculated that one mole of insulin caused the extra trans formation of 13,000 moles of glucose to glycogen per minute by the diaphragm This figure which may, for want of a better term, be called the acceleration number, is an expression, in molecular terms, of the ability of insulin to stimulate a given metabolic reaction In the diaphragm from the hypophysectomized rat, insulin is free of the restraining influence of the pituitary hormones, and one molecule of insulin is more effective in stimulating the formation of glycogen from glucose than in the normal diaphragm resulting in a higher acceleration number (52)

*The type of reaction involved in the combination of* insulin with diaphragm is not unique In 1910, Boehm (53) demonstrated that the alkaloid curarine was able to combine with frog muscle and in this bound form exert its physiological action The phenomenon was strikingly similar to the phenomenon of binding of insulin by rat diaphragm Rothstein (54) has reported that the uranyl ion combines with the surface of the yeast cell, and inhibits the phosphorylation of glucose 2,4-dinitrophenol and epinephrine, but not adrenal cortical steroids, appear to be bound by rat diaphragm *in vitro* (55)

The full significance of the phenomenon of insulin binding by tissue remains to be understood As yet we have no knowledge of the chemical bonds or of the cellular structures involved However, it is possible to speculate that the binding of insulin occurs at specific receptor points in the cell and that the combination of insulin and receptor groups *may be a phenomenon analogous to the* highly specific reactions taking place between antigens and antibodies

The phenomenon of combination of insulin with tissue

is not confined to diaphragm muscle Retroperitoneal adipose tissue has been found to exhibit an increased oxygen uptake in the presence of insulin and such substrates as glucose, succinate or pyruvate (22) As in the rat diaphragm, the insulin effect is obtained after pre treatment of the tissue with insulin followed by washing Insulin also combines with slices of lactating rat mammary gland (56) These findings strengthen the view that the combination of insulin with tissue is an important part of the mechanism of insulin action

The problem of the combination of insulin with tissue is concerned with the mechanism by which this hormone becomes part of cellular structure and is then able to function The exact biochemical mechanism by which insulin influences cellular metabolism has not yet been discovered When all the evidence from studies of the effect of insulin *in vitro* and in the intact animal is considered one fact seems clear—the effect of insulin in increasing the availability of glucose for metabolic reactions is of central importance to the problem of insulin action Many of the effects of insulin can be explained as secondary to an increased metabolism of glucose Any hypothesis of the action of insulin must adequately explain its remarkable acceleration of glucose metabolism

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# INDEX



*This Book*

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